University of Alberta

Teeth and DNA: the mining of Mesolithic and Neolithic teeth in a non-destructive manner.

by

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Abstract

The increase in PCR amplification fidelity has allowed geneticists to gather information about past peoples from human remains. This thesis study examined two ancient populations; Khuzhir-Nuge XIV, a cemetery in Eastern Siberia and various cemeteries in the Iberian Peninsula. Due to poor bone preservation, a new method of mining teeth, that allowed extraction of DNA and did not totally destroy the sample, was tested; then used to analyze ancient samples. At Khuzhir-Nuge XIV the interest was in establishing biological relationships at the cemetery, but poor preservation prevented the analysis of DNA from teeth, and in the Iberian Peninsula ancient DNA could help determine the method of introduction of agriculture to the area, DNA was recovered but it is difficult to determine if the DNA was authentic or contaminant. The mining method is a successful analytical tool with well preserved samples, allowing extraction of DNA with little damage to the sample.

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List of Abbreviations

aDNA	ancient DNA
BAP	Baikal Archaeology Project
bp	base pair
BSA	Bovine Serum Albumin
CDJ	Cement-Dentine Junction
CEJ	Cement-Enamel Junction
CRS	Cambridge Reference Sequence
d.f.	Degrees of Freedom
DNA	Deoxyribonucleic Acid
EDJ	Enamel-Dentine Junction
HVI	hypervariable region I
mtDNA	mitochondrial DNA
µm	micrometer
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
SNP	Single Nucleotide Polymorphism
STR	Short Tandem Repeats
UV	Ultraviolet
YBP	Years Before Present

Chapter 1: Introduction

The history of past societies is chronicled by what they have left behind; artifacts, dwellings and burial grounds of a society can provide information to researchers about the life and death of people that used them. The remains will provide information to researchers in different disciplines: an anthropologist will study a grave and find information about an individual's role in his/her society, an osteologist or geneticist will be able to determine the biological relationships between the people in the cemetery. By combining their knowledge, archaeologists and geneticists are able to answer questions about a society that neither could answer alone.

The gathering of data from past societies using ancient DNA (aDNA) is a relatively new and changing field. Starting with the isolation of DNA by cloning, the advent of PCR technology has allowed the aDNA research field to flourish. Ancient DNA is often used to determine biological relationships; modern and ancient populations can be compared, both within the populations and with each other, to determine how they are related. When the biological data can be correlated with archaeological data, information about social relationships within a society can be extrapolated.

When studying ancient societies, mitochondrial DNA (mtDNA) is the genetic material used by most researchers. The mtDNA is inherited maternally, allowing the study of maternal biological connections within a population. Additionally mtDNA is present in several thousands of copies in a cell, making the retrieval of DNA feasible. The DNA recovered from ancient samples is heavily damaged and challenging to analyze; however, mtDNA provides researchers with a wealth of information.

While mtDNA can be recovered from any cellular part of the body, only a few structures can survive for thousands of years after death. The part of the body that is resistant to degradation is the skeleton, consisting of the bones and teeth. Bone has traditionally been the material of choice for the extraction of ancient DNA when studying past populations (e.g. Meijer, 1992). A drawback associated with the use of bone as a source for DNA is that the surface of bone is porous and prone to contamination with modern DNA. Teeth, an alternative source of DNA, have a smooth surface and are very resistant to contamination. During the lifetime of an individual bone will undergo remodeling and reflect the lifestyle and environment that the person is living in; teeth on the other hand do not undergo any extensive remodeling after their initial formation. Therefore the structure of a tooth closely reflects a person's genetic background with little influence from their environment; this makes teeth particularly useful for the comparison of different populations. Teeth reflect the genetic background of a person as well as select portions of their lives, cementum rings correspond to the age of a person, isotope analysis can reveal where a person was born, spent their childhood and where they spent their adult lives; even the diet of a person can be discovered by examining the wear on their teeth.

To access the DNA in a tooth, it has traditionally been necessary to destroy the whole tooth, unfortunately preventing any other analysis. The information gained from mtDNA is valuable enough to offset the destruction of samples; however, protocols that minimize the destruction of the tooth need to be established. Both the physical examination and DNA analysis of a tooth can be accommodated by using methods of removing the DNA that do not result in total destruction of the tooth.

As DNA technology matures it is becoming more widely recognized and valued in other fields, such as anthropology, for the information that it can provide. The potential of DNA analysis is so great that researchers have included new precautions at their archaeological worksites: disposable latex gloves are worn during excavation and handling of samples, while the teeth and bone destined for DNA analysis are collected before any other samples and stored so that there is no contamination with modern DNA before the samples are analyzed. These precautions ensure that there is a minimum possibility of contamination with modern DNA, and that when the samples gathered are analyzed, the information gained is reliable and the conclusions drawn are sound.

This study will look at a method of removing DNA from teeth that will allow the genetic information to be recovered, while preventing contamination with modern DNA, and damaging the structure of the tooth as little as possible. The mtDNA recovered from teeth using this method will be used to examine the biological relationships of the people from two cemetery populations. One cemetery population is in Eastern Siberia and the cemetery being studied, Khuzhir-Nuge XIV, is being assessed for amplifiable DNA from the teeth. The bone from this cemetery has been worked with for six years with no successful retrieval of DNA. Teeth have the potential to preserve DNA after the bone has been degraded and Khuzhir-Nuge XIV will be used to determine if the DNA in the teeth is better preserved than the DNA in the bones at this cemetery. The second cemetery population is from the Iberian Peninsula and consists of several Mesolithic and Neolithic sites tested to determine if there is amplifiable DNA present.

The sites studied and a brief background of their respective histories will follow in Chapters 2 and 3; the areas covered are respectively Khuzhir-Nuge XIV and the Iberian Peninsula. Following the introduction of the study sites, the structure, damage and preservation characteristics of DNA will be discussed in Chapter 4.

Understanding the material chosen for DNA extraction is essential, therefore the growth, structure and diagenesis of teeth are explored in Chapter 5. Preceding a description of the mining method used for teeth during this study is Chapter 6, a review of the other protocols in published literature for different techniques used to remove DNA from teeth.

Chapter 7 will be a description of the mining method developed as part of this study, as well as a description of the other methods and materials used. The results of the study of DNA removal from teeth, modern teeth, as well as teeth from ancient Khuzhir-Nuge XIV and the Iberian Peninsula, are presented in Chapter 8 along with a short discussion. Chapter 9 finishes the thesis with conclusions about the study and recommendations for future endeavors regarding teeth as a source of DNA.

Chapter 2: Khuzhir-Nuge XIV

The Baikal Archaeology Project (BAP) is a large international multidisciplinary project that is based at the University of Alberta (Edmonton, Canada) and the University of Irkutsk (Irkutsk, Russia). The BAP is researching the lives and deaths of the prehistoric hunter-gatherer cultures that lived in the Cis-Baikal region of Eastern Siberia 8000 to 3000 years ago. There were two distinct cultures that inhabited the area around Lake Baikal, the Kitoi and the Serovo-Glaskovo. This time period and these populations are of particular interest to researchers due to the large number of identified cemetery and habitation sites. Because hunter-gatherer cultures tend to leave little archaeological evidence of their presence, the sites in the Cis-Baikal region are a unique resource that will allow unprecedented research about their lifestyle. The change in cultures from hunter-gatherer lifestyles to agriculture and pastoralism are often attributed to climate change and marginalization due to an influx of other agrarian cultures; in the Lake Baikal region there was no climate change or incursion of agrarian cultures to account for the change from the hunter-gatherer lifestyle. The climate and altitude are very similar to that experienced in parts of Canada; the average summer temperature is 20 °C and the average winter temperature is – 26 °C (Weber *et al* 2002). By examining the populations of Eastern Siberia there are many parallels that can be drawn between the Russian populations and the aboriginal Canadians.

BAP comprises five different modules encompassing different research fields working together and combining their knowledge. By working together and sharing their information the researchers in the human genetics, human osteology, archaeological context, environmental context and ethnographic context are able to pool their knowledge of the Kitoi and Serovo-Glaskovo populations; the combined results from these modules will provide more information about these past populations than any single discipline could.

One of the major foci of the BAP has been to study an observed hiatus in the preserved mortuary practices between the occupation of the Baikal region by the Kitoi and the Serovo-Glaskovo. The region was inhabited by the Kitoi starting at 8000 years before present (YBP). There is an approximately 800 year gap from 6100-5300 YBP during which there was little archaeological evidence preserved of human habitation in the region. This lack of artifacts is due to a combination of changes in mortuary practices and a smaller population. There is evidence that the Serovo-Glaskovo inhabited the region after this hiatus until 3300 YBP (Weber and Bettinger 2002). Differences in subsistence strategies and population structure suggested that these two cultures were discrete groups rather than a single population that changed over time; genetic analysis has shown that this is true and that the Kitoi and the Serovo-Glaskovo were not biologically related populations. The Serovo-Glaskovo had a large population divided into a large number of small groups that moved throughout the Cis-Baikal region. A diverse diet ensured enough food for the Serovo-Glaskovo and the food was shared equitably as evidenced by the balance in the number of males and females in each age group. The Kitoi had a smaller population than the Serovo-Glaskovo, and a more limited diet high in fish. With the limited diet the Kitoi suffered occasional, non life-threatening, food deficiencies.

A part of this thesis involves analysis of aDNA from a Serovo-Glaskovo cemetery, Khuzhir-Nuge XIV, on the western shore of Lake Baikal (Weber 1995). This community cemetery was used by the Serovo-Glaskovo about 3000 years ago and contains 86 graves, all of which have been excavated. Khuzhir-Nuge XIV was used at the end of the Serovo-Glaskovo occupation of the Cis-Baikal region and might provide information about why the Serovo-Glaskovo disappeared from the region. Bone and teeth samples were collected at the time of excavation and stored in an anthropological collection; not intended for DNA analysis the tooth samples were collected without precautions against contamination. Visual inspection showed that the bone and teeth samples were poorly preserved, with many of the teeth containing fractures and crumbling when they were handled. Attempts to extract DNA from bone samples for the past six years have been unsuccessful, teeth have been chosen as another material that has potential to provide genetic information. Because DNA within teeth is protected from degradation there is potential to extract amplifiable DNA even if there is no DNA detectable within the bone. Therefore part of this study was testing if there is amplifiable DNA present in teeth when it has been demonstrated that there is no amplifiable DNA in bone.

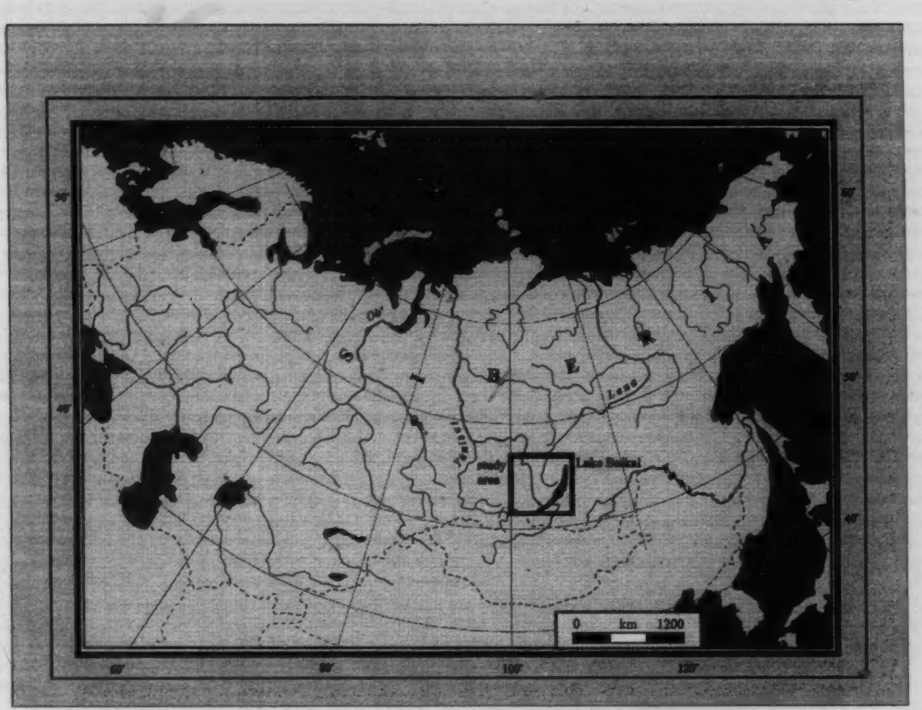
study area
Lake Baikal
Lena
0 km 1200
60°
80°
100°
120°

Figure 2.1a: Map of Russia Highlighting Cis-Baikal Region

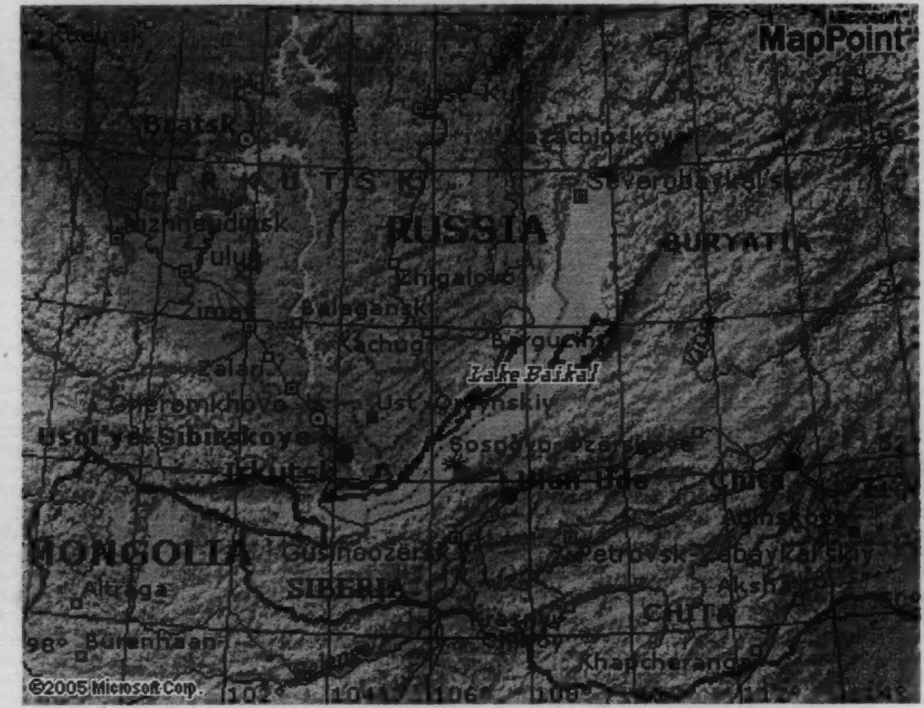
MapPoint
Bratsk
IRKUTSK
RUSSIA
BURYATIA
Zhigalovo
Balagansk
Kachug
Lake Baikal
Cheremkhovo
Usol'ye-Sibirskoye
Irkutsk
Ulan-Ude
Chita
MONGOLIA
SIBERIA
CHITA
Khapcheranga
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Figure 2.1b: Map of the Cis-Baikal Region containing Lake Baikal

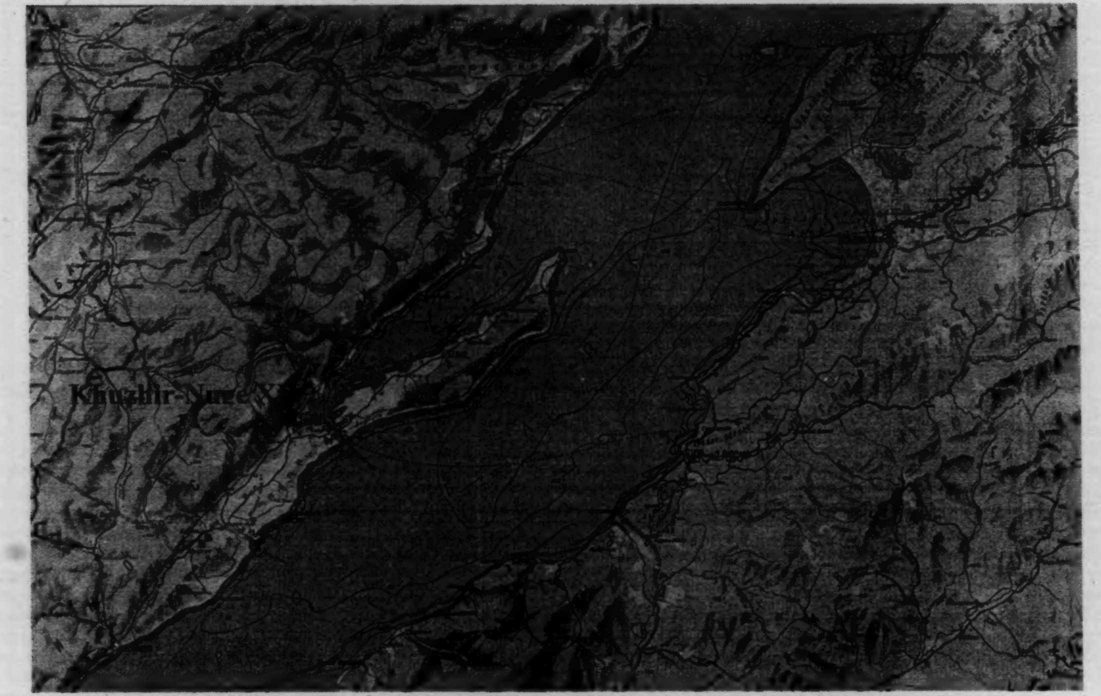


Figure 2.1c: Map of Lake Baikal showing the cemetery Khuzhir-Nuge XIV

The biological examination of Khuzhir-Nuge XIV can address several different issues regarding the hunter-gatherer populations of the Cis-Baikal region. There is information from an earlier Serovo-Glaskovo cemetery, Ust-Ida, that was used shortly after the hiatus ended approximately 5300 YBP. The comparison of mtDNA haplogroup information, motifs of informative polymorphisms in DNA, from the early and late period of the Serovo-Glaskovo inhabitation can provide information about where the population migrated from, how the population changed during their occupation of the Cis-Baikal region and where the Serovo-Glaskovo moved to when they left the area. The possibility of a biological continuity between the Kitoi and the Serovo-Glaskovo was explored in a previous study of the Baikal Archaeology Project as a portion of the PhD thesis work of Dr. Karen Mooder (Mooder 2004). The results of mtDNA haplogroup analysis of cemetery populations from the Kitoi and the Serovo-Glaskovo indicate that the two groups were distinct and not biologically continuous (Mooder 2004). However, the addition of samples from another Serovo-Glaskovo cemetery will increase confidence in the conclusion of population discontinuity between the Kitoi and the Serovo-Glaskovo. The comparison of mtDNA haplogroups of individuals buried in the same grave can reveal if there are any matrilineal relationships between the individuals in the same grave. Finally information gained form Khuzhir-Nuge XIV would confirm that it is possible to retrieve DNA from teeth where there is no amplifiable DNA detected in the bones.

Chapter 3: Iberian Peninsula

One of the questions that genetics may be able to help answer is about the introduction of agriculture to the Iberian Peninsula. Farming appeared in the archaeological record of the Iberian Peninsula at the same time as the Western Mediterranean at around 5400 YBP (Zilhão 2001). The method of introduction of agriculture to the Iberian Peninsula is contentious. The main theories are demic diffusion (Ammerman and Cavalli-Sforza 1984), which proposes that there was an influx of a genetically distinct population and agriculture practices from the Middle East. The other main theory is the cultural diffusion model (Zvelebil 2000), by which ideas and practices were transmitted without significant exchange of genes, and the last theory is an indeterminate mixture of these two models, which suggest that there was an exchange of both technology and genes. In order to give a clear idea of what happened, it is necessary to combine the information from different fields: archaeology, archaeozoology, osteology, genetics and linguistics (Diamond and Bellwood 2003).

Zilhão argues that the patterns seen in the archaeological evidence support the demic diffusion model, with the colonizers arriving by sea (Zilhão 1993; 2000). While the presence of distinct areas such as the Estremadura, where there was a largely terrestrial diet, suggested to Zilhão that these areas were colonized by farmers. The intervening area, such as the Tagus estuary, where a diet high in marine resources was consumed, is where the hunter-gathers remained (Lubell *et al.* 1994).

That very same evidence is interpreted differently by Lubell *et al* (1994). He concludes that there is a marked difference in diet between the Mesolithic and Neolithic populations, but that this was the intensification of a trend that had started in the Mesolithic, not due to the replacement of the population. Further support for the lack of replacement was found by examining the crania of the Mesolithic and Neolithic populations. When the all available samples were studied there was no evidence of genetic discontinuity based on non-metric traits; instead there was a slow trend of change that started before the Neolithic (Jackes *et al.* 1997).

Examination of aDNA from samples in the Iberian Peninsula region may give information that will help to clarify whether the transition to agriculture was due to demic diffusion, cultural diffusion, or both. The extraction of DNA for analysis from Mesolithic and Neolithic skeletal remains from several sites in the region would provide the information needed to help confirm or refute the theories regarding the introduction of agriculture to the Iberian Peninsula. Comparison of the mtDNA haplogroups of the populations, when compared with each other and with modern populations, may be able to distinguish if there was a change in mtDNA haplogroups and haplogroup frequencies between the known hunter-gatherer cultures and the agrarian cultures. Changes in the mtDNA haplogroups seen would indicate that people from a different population had moved to the region.

The mtDNA haplogroups of Europe have been well studied (Torrini *et al.* 1998). There is a southeastern to northwestern gradient for many European mtDNA haplogroups. The clinal gradient has been interpreted as a demic diffusion from the Near East to Europe. The majority of the haplogroups seen in Europe had European founders that date to pre-Neolithic times and were not a result of the demic diffusion from the Near East. 15% of the mtDNA haplogroups in Europe have been dated to Neolithic times (Richards *et al.* 1996), implying that there was a limited amount of demic diffusion prior to the Neolithic. This greatly reduces the impact of a demic diffusion during the spread of agriculture between the Mesolithic and Neolithic because there continues to be gene flow into the Iberian Peninsula after the Neolithic. Therefore, to determine if there was population replacement in the Iberian Peninsula it is better to compare the Mesolithic haplogroups to the Neolithic haplogroups rather than the modern population. Changes in the mtDNA haplogroups seen and their frequencies should reveal if there is a discontinuity between the Mesolithic and Neolithic samples. In the Iberian Peninsula a haplogroup that might be diagnostic of change is Haplogroup J. This haplogroup originated in the Near East and diffused into the Iberian Peninsula reaching Europe at least 10 000 years ago. In the modern populations of the Iberian Peninsula haplogroup J is very rare (Richards *et al* 1998). The timing of the introduction of haplogroup J to the Iberian Peninsula could help determine the mode of introduction of agriculture to the area. If haplogroup J is found after the introduction of agriculture, then haplogroup J and farming may have moved into the Iberian Peninsula at the same time. Currently there has been no haplogroup J detected in the bones and teeth from the three Neolithic or seven Mesolithic individuals that have been studied by Chandler and colleagues (Chandler *et al* 2005). However, if there is a difference between the other mtDNA frequencies in the Mesolithic and Neolithic samples, then there may have been a genetically distinct population that brought farming with it. One of the possibilities is that farmers from the Mediterranean moved along the coastline to reach the Iberian Peninsula (Zilhã0 2001). If there is no significant difference detected in mtDNA haplogroups between the Mesolithic and Neolithic, it supports the cultural diffusion model and there may only have been a cultural exchange that resulted in the introduction of agriculture.

There were eight Mesolithic and Neolithic sites in this study, a mixture of archaeological areas. Three sites are caves that were used exclusively as ossuaries and one that was a shell midden (Lubell *et al* 1994). At Feteira, a Neolithic ossuary in a limestone cave, the bones were covered with calcium carbonate, potentially adversely affecting the ability of hydroxyapatite to bond and preserve the DNA (Jackes *et al* 2001). One site, La Garma, contains both Mesolithic and Neolithic remains. These sites were chosen as test cases. Once it is verified that there is amplifiable DNA that can be retrieved from these sites without total destruction of the sample, then a larger number of samples can be worked with to produce statistically significant results.

Chapter 4: Ancient DNA Analysis

4.1 DNA

Deoxyribonucleic Acid (DNA) is a double helical structure that encodes all the genetic information to grow and maintain a human body. There are four bases: the purines, adenine and guanine; and the pyrimidines, cytosine and thymine. Adenine forms pairs with thymine and guanine pairs with cytosine; this predictable pairing makes it possible to determine the sequence of both complementary strands of the DNA molecule.

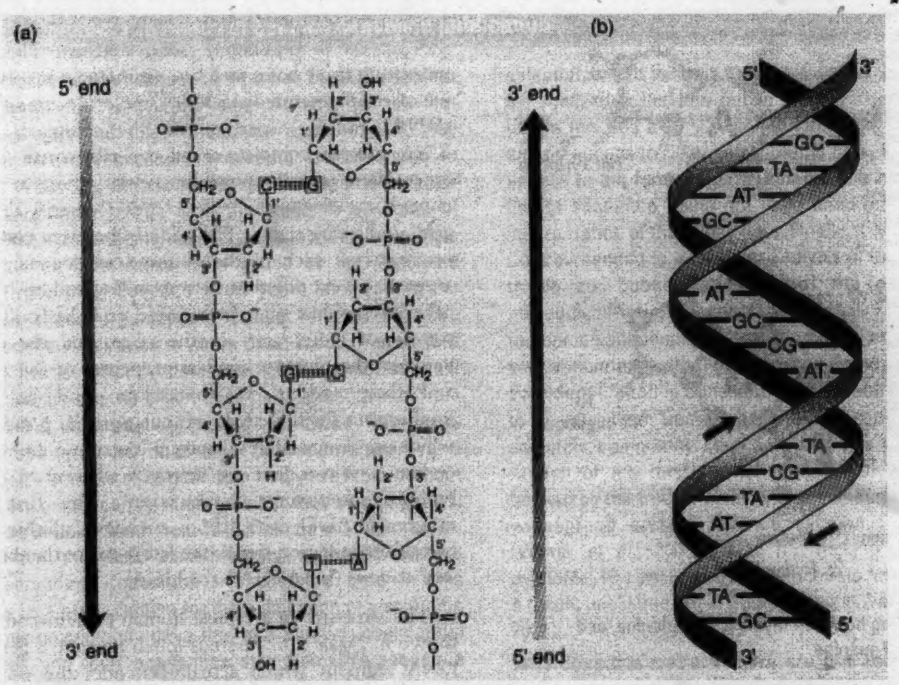


Figure 4.1: The structure of DNA, a) showing the anti-parallel strands and b) the double helix structure. (Jobling *et al* 2004)

There is DNA in every nucleated cell in the human body. In each of these cells there are two different and distinct genomes; the nuclear genome which is present in two copies in every cell and the mitochondrial genome that is present in hundreds of copies in every mitochondrion and thousands of copies in every cell (Giles *et al* 1980 and Wallace

1995). The circular mitochondrial DNA is 16 569 base pairs (bp) long and encodes proteins used in mitochondrial functioning (Anderson *et al* 1981); it is a fraction of the size of the nuclear genome. Both the nuclear DNA and mitochondrial DNA (mtDNA) contain markers that have been characterized and used to study human populations (e.g. Kivisvild *et al* 2002); the hypervariable regions in mtDNA, the Y chromosome STRs, (short tandem repeats) in nuclear DNA and SNPs (single nucleotide polymorphisms) in nuclear DNA are examples of this. STRs are short, 2-10 bp, motifs that are repeated. The number of times a STR repeats can be counted and characterized for individuals. SNPs are single polymorphisms at known locations and can be used to identify individuals as well.

Ancient DNA (aDNA) is degraded and consequently difficult to work with. Mechanisms of degradation will be discussed later in this chapter. When aDNA is being examined mtDNA is commonly chosen for study. Mitochondrial DNA is present in thousands of copies in every cell, and compared to the two copies of nuclear DNA, there is an increased likelihood of detecting an amplifiable fragment of mtDNA. With thousands of copies of mtDNA in a cell, not only is it likely that some will not be degraded, it is also likely that some will remain that contain the portion of the mtDNA that the researcher is interested in without any of the damage from diagenesis that can mimic polymorphisms. The DNA polymorphisms are used to determine population affinities in ancient and modern populations (Kivisvild *et al* 2002). These DNA polymorphisms are referred to as haplogroups. Haplogroups can be used to identify individuals and maternal relationships as well as population relatedness.

The usefulness of mtDNA in determining maternal lineages is due to some interesting properties of mtDNA. MtDNA is maternally inherited, has a rapid mutation rate and very limited recombination. The mtDNA is inherited almost exclusively from the mother (Giles *et al* 1980) and it is very rare for paternal mtDNA to be found in offspring (Shwartz and Vissing 2002). The mitochondria and mtDNA are passed from the mother to the offspring through the egg at fertilization. There is some mtDNA in the sperm that can enter the egg (Ankel-Simons and Cummins 1996), but this is rapidly degraded after fertilization (Shitara *et al* 2000). Even if there is a rare case of paternal inheritance of mitochondria, the majority of the mtDNA found in tissue samples is maternal. MtDNA does not regularly undergo recombination thereby providing a clear record of maternal lineage (Eyre-Walker and Awadalla 2001). There are nuclear pseudogenes, copies of mtDNA genes in the nuclear genome, which may rarely allow recombination between the nuclear and mitochondrial genome to occur (Kamimura *et* al1989), but this is currently unproven. This lack of recombination in mtDNA and maternal inheritance allows the distinction of matrialineages and the identification of maternal relationships among individuals. Conversely when nuclear DNA is inherited, it undergoes recombination and it is difficult to distinguish relationships beyond a few generations. The Y chromosome, part of nuclear DNA, does not undergo recombination (there is only a single copy in phenotypically normal males) making the Y chromosome helpful in the determination of paternal relationships when there is sufficient preservation to allow the amplification of nuclear DNA.

Nuclear DNA is organized into 46 chromosomes; there are 22 pairs of autosomes and 2 sex chromosomes. The mitochondrial genome is much smaller than the nuclear genome and is a single circular piece, 16 569 bp long, while there are 3 x 10^9 bp in the nuclear genome. The mitochondrial genome was first sequenced in 1981 (Anderson *et al* 1981) and revised in 1999 to correct some errors and confirm some rare polymorphisms (Andrews *et al* 1999). The Cambridge Reference Sequence now serves as a basis for the identification of haplogroups, which are used to determine maternal relationships. While the mitochondrial DNA has both coding and non-coding regions, the majority of the mitochondrial DNA consists of coding regions, which are highly conserved since the proteins it encodes are essential for functioning of the mitochondria. The non-coding region is called the D-Loop (displacement loop) and, as it is not essential that the D-loop region remain conserved, there is no selection against mutations, which leads to a high rate of mutation within the D-loop. The mutation rate of mtDNA is ten to twenty times greater than that of nuclear DNA (Wallace *et al* 1987). This mutation rate is due to the conditions in the mitochondria. There are oxidizing chemicals present that can damage the mtDNA and there are fewer enzymes involved in the repair of the mtDNA, both after damage and during replication. There are several distinct regions within this D-Loop: Hyper-Variable Region 1 (HVI), Hyper-Variable Region 2 (HVII) and Hyper-Variable Region 3 (HVIII). The polymorphisms within the three regions have been studied and characterized. HVI has the highest variability at 26% (88/342 bp), HVII has a variability of 24% (65/268 bp), and the HVIII variability of 18% (21/137 bp) (Lutz *et al* 2000). The HVI is the most informative region and most often used for determining haplogroups. If more information is needed it is possible to distinguish similar HVI haplogroups using polymorphisms from HVII. The polymorphisms in HVIII are currently the least studied, but there is enough information available to distinguish between very closely related haplogroups. These motifs of polymorphisms within the hypervariable regions form the basis for haplogroups that allow the separation of populations into distinct mitochondrial lineages.

4.2 Mitochondrial DNA Haplogroups

The mutations seen in the hypervariable regions of the mitochondrial genome accumulate over time. The mutations occur in motifs, and these motifs are characterized and named as specific haplogroups. Over time new haplogroups have evolved from the base motif of the original haplogroup and as populations have migrated they developed their own distinct haplogroup frequencies. All haplogroups are related to each other and it is possible to determine which haplogroups evolved first (Cann 1987). The differences and similarities between the haplogroups tell researchers how closely related the haplogroups are to each other and therefore how long ago the haplogroup originated. It is possible to determine the time when a haplogroup originated by examining the mutations that haplogroups have in common, when the oldest mutations in the haplogroups are identified it is possible to use the mutation rate of mtDNA and extrapolate to when the haplogroup originated. Cann *et al* in 1987 studied the mtDNA of placentas from 145 women from the United States, Australia and New Guinea. The study also included two cell lines, one from a Black American and the other from an aboriginal South African; as well as including Africans, Asians, Caucasians, aboriginal Australians and aboriginal New Guineans. Using restriction digestion mapping the haplogroup motifs for each sample were identified. The researchers concluded that the mtDNA lineages that they discovered contained two branches; one branch was African and the other branch contained all the other populations with the whole tree rooted in the African lineages. Therefore it was postulated that the most parsimonious origin of humans was in Africa. There was also evidence that there were multiple mitochondrial lineages that contributed to the colonization of the different regions of the world rather then a single group.

By studying the geographic distribution of a haplogroup it is possible to distinguish where the haplogroup originated. The population with the highest incidence of the haplogroup is most likely to be the population where it originated. It is chance and circumstance that cause a haplogroup to become prevalent in a population. One circumstance that can lead to a haplogroup becoming more prevalent is the reproductive isolation of a population; an example of this is the origin of haplogroup V in the Iberian Peninsula. Torroni *et al* (1998) looked at the distribution of haplogroup V, which is limited in its geographical distribution and had an estimated origin 12,200 years ago. During the second pleniglacial period human populations retreated into refuges, one of which was the Iberian Peninsula of Western Europe. At the end of the second pleniglacial period the human population left the glacial refuge and as the population spread the haplogroup V spread across Europe.

There are a variety of haplogroups that are present in every population, but each population contains only a subset of the total number of haplogroups from around the world. Within a geographic area there are often distinct populations. These smaller populations may contain only a subset of the possible haplogroups. Using the frequency and presence of haplogroups it is possible to determine if populations are related to each other (Torrini *et al* 1998).

4.3 Asian mtDNA Variation

The Eastern Siberian population from the Khuzhir-Nuge XIV cemetery that ass examined in this study is expected to exhibit Asian haplogroups. Asian haplogroups are divided into two large groups that are called macro- or super-haplogroups (Kivisild *et al* 2002); these super-haplogroups are referred to M and N. The super-haplogroup M contains the haplogroups C, D, G, G2a and Z, super-haplogroup N contains the haplogroups A, B, X, F and Y. A summary of the Asian haplogroups and the HVI substitutions that define them is shown in table 4.1. Because only HVI polymorphisms were examined in this study, HVII polymorphisms are not presented.

There is a pattern to the geographic distribution of the Asian haplogroups which reveals information about the population history (e.g. Kivisvild *et al* 2002). The most heterogeneous portion of the population is found in central Asia and includes those living on the steppes in Siberia. The southern portion of Asia has high frequencies of the haplogroups B and F, while northern Asia has haplogroups A, C, D, G, X, Y, and Z predominating. Central Asia, because it is most similar to Siberia in the type and number of haplogroups that are found there, is likely to be the region from which the Asian populations were founded (Kolman *et al* 1996).

Table 4.1: The Characteristic Substitutions of Asian mtDNA Lineages, there are additional substitutions that are built on these backgrounds. All substitutions noted here are transitions. (Kivisild *et al* 2002)

Haplogroups		HVI Substitutions						
M								
	C	16223						
	D	16233	16298	16327				
	G	16233	16362					
	G2a	16017	16227	16278	16362			
	Z	16129	16185	16223	16224	16260	16298	16519
N		16223						
	A	16223	16290	16319				
	B	16189	16217					
	X	16189	16223	16278				
	F	16304						
	Y	16189	16266	16159				

4.4 Iberian mtDNA Variation

The samples from Iberian Peninsula that are examined in this study contain typical Western European haplogroups. The haplogroups found in the modern day Iberian Peninsula include members of the haplogroup families H, I, J, K, L, M, T, U, V, W, and X (Pereira *et al* 2000). Haplogroup H is the Cambridge Reference Sequence (CRS) that was the first human mtDNA sequenced (Anderson *et al* 1981). All other haplogroups are defined as differences from the CRS. A summary of the haplogroups found in the Iberian Peninsula and the polymorphisms that are indicative of those haplogroups is shown in table 4.2.

The distributions of these haplogroups vary across Europe. It is uncommon in most western European populations except the Basque, yet one of the highest frequencies of haplogroup V is found in the Sami of northern Europe (Sajantila *et al* 1996). Haplogroup J arrived in Europe 10,000 years ago (Richards *et al* 2000), having originated many years before that. The highest frequency of haplogroup J is in the eastern Mediterranean (Richards *et al* 1998) and is very rare in the modern Iberian Peninsula.

Table 4.2: The Characteristic Substitutions of Iberian mtDNA Lineages, there are additional substitutions seen that are built on these backgrounds. All substitutions noted here are transitions. (Pereira *et al* 2000)

Haplogroups	HVI Substitutions	
H	CRS	
I	16129	
J	16069	16126
K	16224	16311
L	16223	
T	16126	
U	CRS	
V	16298	
W	16223	16292
X	16189	16278

4.5 Influences on the Preservation of DNA in Tissue

While every nucleated cell in the human body contains DNA, not all the tissues of the body are preserved equally well after death. The degree of degradation of DNA immediately following death is dependent on tissue type. The soft tissues of the body, such as muscles and organs, have high water content and the DNA can be oxidized rapidly. Additionally neighboring cells release degrading enzymes that affect the surrounding cells (Bar *et al* 1998, Hochmeister *et al* 1991) and cause the surrounding tissue to be degraded. The hard tissues of the body degrade much slower than the soft tissues. Teeth and bones will survive longer than any other part of the body. The degradation of teeth will be discussed in greater detail in Chapter 5. Hard tissues tend to be the material of choice when isolating ancient DNA due to their better long term preservation (e.g. Keyser-Traqui *et al* 2003, Gilbert *et al* 2004). Hard tissues are preferred for their low water content and high hydroxyapatite content which prevents degradation of the organic components; both bone and teeth are used for the isolation of DNA. When trapped in the dry highly mineralized tissue of teeth and bone, DNA can possibly survive the post-mortem interval. The preservation of DNA, collagen and cellular structures suggest a process of individual mummification of each cell; surrounded by hard tissues these cells and their DNA are protected from microorganisms and other physical agents (Hummel and Herrmann 1994).

DNA can be found in hard tissues for hundreds of years postmortem (Ovchinnikov *et al* 2000). One of the reasons that DNA persists is because DNA will bind to hydroxyapatite where it cannot participate in the chemical reactions that cause degradation (Hochmeister *et al* 1991). Bone is composed of 70% hydroxyapatite, while the enamel of teeth contains 98% hydroxyapatite. The chemical formula of hydroxyapatite is $Ca_{10}(PO_4)_6(OH)_2$. Hydroxyapatite contains many sites that can bind to negatively charged molecules (Betts *et al* 1981), such as carbonate, which binds to the surface of the apatite crystals. The highly negative charge of the phosphate backbone of DNA binds strongly to the positively charged hydroxyapatite, occupying the spaces that carbonate would normally bind to. This strong binding appears to confer a resistance to further postmortem damage of the DNA by making the DNA inaccessible to degrading agents. When DNA is bound to hydroxyapatite the rate of depurination, a common cause of DNA degradation, decreases by 50% (Lindahl 1993).

There are other factors besides the binding of DNA to hydroxyapatite that can be used as a guide for determining optimal sources of intact DNA. The use of different types of bone as a source of DNA has been studied; bones that are composed primarily of compact bone, such as a femur, appear to protect the DNA well (Henderson 1987). However, the yield of DNA from spongy bone can be 10 to 20 times higher than from compact bone (Lee *et al* 1991). Cells that are highly isolated, such as the osteons in compact bone, are not subject to the same degree of enzymatic destruction as cells in soft tissue. The optimal solution would be to extract DNA samples from a soft bone that is encased in compact bone; vertebrae are an excellent example of spongy bone that is protected by compact bone.

The DNA in the interior of teeth is protected from degradation by the hard enamel encasing the soft tissue inside. 98% of the tooth enamel is inorganic hydroxyapatite; however there is no DNA in the enamel of teeth for the hydroxyapatite to bind (Hillson 1996). The 2% organic content of enamel is the protein amelogenin and is acellular. The dentine tissue in the interior of the tooth, which is protected by the exterior enamel and cementum, has a hydroxyapatite content of about 25% (Hillson 1996). Despite the smaller volume of a tooth compared to the amount of bone used in DNA extraction, there is a high concentration of DNA within the tooth. A comparison of DNA extracted from a piece of jawbone and a tooth demonstrated that there was 5 times as much DNA extracted from a single tooth as there was from a similar portion of the jawbone (Benedetto *et al* 2000). In some earlier studies the unerupted teeth of children were preferred as a source of DNA; there is no apical foramen through which contamination could enter the pulp cavity of the tooth (Drancourt *et al* 1998). Since then there has been a shift to using adult teeth, which are generally more available, and in which there is a greater volume of dentine to use for extraction (Gilbert *et al* 2004).

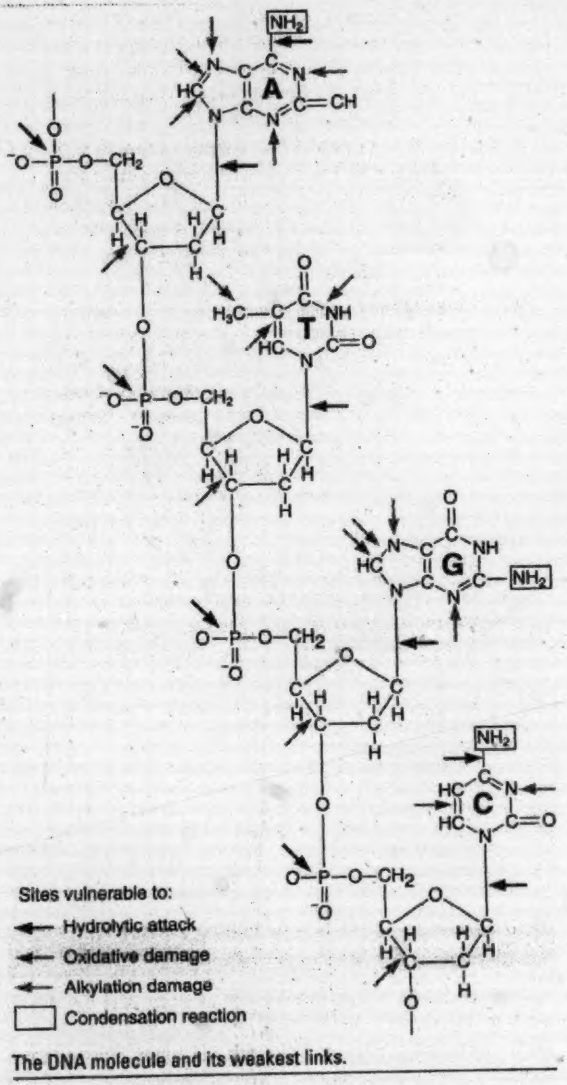
Even when choosing between erupted adult teeth for DNA analysis the youngest tooth is the best choice. The molars are the last teeth to erupt and the second molar erupts after the first molar, meaning that the second molar is used less for chewing than the first molar. Because the act of chewing over a life time wears down the tooth, the tooth will produce more dentine to protect the pulp chamber. This pattern of sclerotic deposition can be used to determine the age of an individual. The dentine deposited in response to wear is referred to as sclerotic dentine. Because there is no division of odontoblasts during sclerotic deposition the amount of DNA does not increase. The third molar emerges last, but it tends to have a non-uniform shape and size; making the third molar less useful in a population study than the second molar. The third molar is the most common tooth not to emerge; the instance of agenesis can reach 20% in the population (Kolenc-Fusé 2004). The second and third molars are firmly rooted within the jawbone and are retained *in situ* even if the other teeth have been moved out of the jaw. The best tooth to use for extraction is a second molar from a young adult individual without any caries.

4.6 The Chemistry of DNA

DNA, both in the body and in the environment, is constantly subject to damage; while a cell is healthy, the DNA is quickly repaired by cellular mechanisms (Lindahl 1993, 1996). These mechanisms work together to repair the damage that occurs to DNA through the by-products of cellular metabolism and the damage produced by exposure to the environment. When the cell dies the repair mechanisms no longer work and any damage done to the DNA remains. Consequentially ancient DNA is damaged and fragmented. There is immediate damage to DNA when the cell dies caused by the degrading enzymes and additional damage that accumulates over time after the death of the cell. The additional damage that occurs in DNA after death is caused by hydrolysis and oxidation (Rogan and Salvo 1990, Lindahl 1993, 1996).

Immediate damage to the DNA occurs as a result of autolysis after death. When an organism dies the cells of the body receive apoptotic signals and degradation will occur. Because there is no longer the intake of fresh nutrients or the removal of waste occurring, cells accumulate toxic materials and will die. The soft tissues are quickly affected by the release of hydrolytic enzymes contained in the lysosomes (Bar *et al* 1998); the enzymes released by the lysosomes first kill the cell and then attack neighboring cells. The soft tissues are the first to degrade, with the released enzymes degrading the abundant organic matter, while the hard tissues take longer to degrade. When cells in hard tissues are highly isolated, such as the osteons in compact bone, they are not subject to the same degree of enzymatic destruction as cells in soft tissue and often survive longer than any other cell in the body.

Hydrolysis cleaves bonds within the DNA molecule. The glycosyl bond that connects bases to the phosphodiester backbone of the DNA and the phosphodiester bonds of the DNA backbone are vulnerable. When the glycosyl bond joining the ribose sugar to the purine is cleaved it is called depurination, the purine bases are guanine and adenine (Lindahl 1993). When the base is lost, the DNA molecule is vulnerable to fragmentation. The fragmentation due to hydrolysis can also occur as the phosphodiester bonds that form the backbone of the DNA molecule are broken (Eglinton and Logan 1991, Lindahl 1993). Once depurination occurs the whole DNA strand can be fragmented; initially only part of the backbone of the DNA is cleaved and this is called a single stranded break, cleaving half of the phosphodiester backbone leaves the other portion vulnerable. A double stranded break will allow the broken portions of the DNA molecule to disassociate from each other, making analysis of the DNA molecule difficult. Lindahl and Anderson (1972) discuss the rate of depurination and double-stranded breakage, when they were working with the double stranded covalently closed circular DNA of the phage PM2. At 37°C there is a single depurination event after 2000 hours in neutral phosphate buffer. The presence of acidic conditions, increased temperature and Mg^{2+} ions, all increase the rate of depurination under *in vivo* physiological conditions. Chain cleavage at apurinic sites proceeds slowly under physiological solvent conditions but when cell extracts are added to the solution there is very rapid breakage of the DNA molecule at apurinic sites. Within cells some enzymes involved in repair specifically attack apurinic sites that are present in cells. These enzymes use an excision- repair mechanism that is also used in the repair of pyrimidine-dimers.


NH2
A
T
G
C
Sites vulnerable to:
Hydrolytic attack
Oxidative damage
Alkylation damage
Condensation reaction

The DNA molecule and its weakest links.

Figure 4.2 DNA Structure and Damage Sites (Jobling *et al* 2004)

Hydrolytic changes in bases can cause miscoding during PCR amplification that mimic premortem mutations. The deamination of cytosine changes the base to a uracil, which is normally found only in ribonucleic acid (RNA), where it will pair with thymine. When the deaminated cytosine is used as a template in PCR amplification it will change from a C-G pairing to an A-T pairing (Lindahl 1993, Hoss *et al* 1996, Hofreiter *et al* 2001). During the first round of replication the polymerase will recognize the uracil and will incorporate an adenine into the complementary strand. In subsequent amplifications the adenine will act as a template for incorporation of a thymine in the complementary strand.

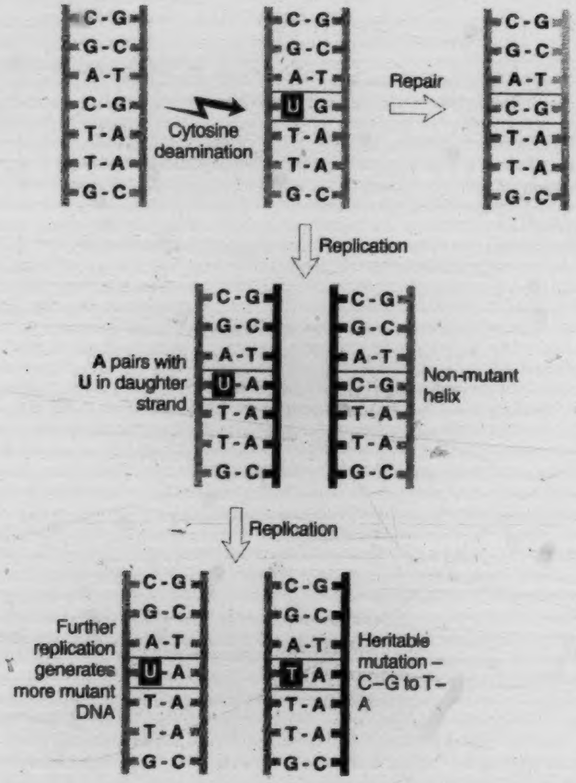

Cytosine deamination
Repair
Replication
A pairs with U in daughter strand
Non-mutant helix
Replication
Further replication generates more mutant DNA
Heritable mutation – C–G to T–A

Figure 4.3 DNA Damage leading to Mutation.
(Jobling *et al* 2004)

Oxidative damage can also occur as a result of ionizing radiation and the free radicals produced by ionizing radiation. The pyrimidine bases, thymine and cytosine, are subject to alteration by ionizing radiation. One of the most common examples of this is the dimerization of thymine through exposure to ultra-violet (UV) radiation (Ou *et al* 1991). Pyrimidine dimers disrupt the replication and repair of DNA. Adenosine and guanine are generally oxidized by the free radicals produced by ionizing radiation through interaction with water molecules (Lindahl 1993). The main product of this type of damage is 8-hydroxyguanine. Levels of 8-hydroxyguanine may be indicative of the amount of oxidative stress in tissues.

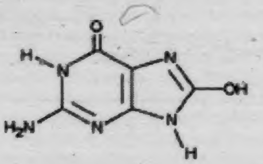


Figure 4.4 8-hydroxyguanine

DNA containing apurinic sites may be incorporated into a humic acid-like compound, melanoidine, by the Maillard reaction. When bound in a compound like this, DNA is theoretically resistant to further hydrolysis (Bada 1998). However, the DNA may also be resistant to interaction with the polymerase of PCR; before the DNA can be analyzed it must be removed from the melanoidine with the reagent N-phenacylthiazolium bromide (PTB). Poinar *et al* (1998) have used PTB as an addition to the extraction buffer to recover DNA where it was previously inaccessible to study, and a sample that was thought to have no DNA present was shown to contain amplifiable DNA.

Many of the same conditions that degrade DNA will also degrade collagen, the organic component of teeth and bones. When collagen has been degraded it is very likely that its associated DNA has been degraded as well. The degree of degradation of collagen can be assessed more readily than the degree of degradation of DNA. The simplest way to assess degradation of collagen is to observe the sample; if the sample is crumbling, then the collagen and the DNA is likely to be degraded as well. Due to the correlation between DNA degradation and collagen degradation it is possible to inspect samples visually to determine their suitability for DNA extraction. One of the results of degradation is that the DNA will be very fragmented. This requires that the amplification primers are designed to amplify DNA fragments that are 200 base pairs or shorter. Even when taking fragmentation into account and working with short pieces of DNA, damage will accumulate over time and it is unreasonable to expect fragments of DNA of a analyzable length to survive for more than 10 000 years in temperate environments and 100 000 years when cold (Lindahl 1993, Poinar *et al* 1996).

4.7 DNA and the Environment

After death DNA is no longer protected from damage as there is a complete loss of the mechanisms that repair damage. Environment that was previously of little concern is now vital to the preservation of intact DNA. Damage to DNA is governed by the same laws that apply to all chemical reactions. The temperature, altitude, water and radiation to which DNA is exposed will affect the amount of DNA damage and therefore, the ability to gather information from the DNA.

Temperature affects the speed of chemical reactions in a predictable manner; there is a doubling in the reaction rate for every 10°C increase in temperature above 0 °C (Von Endt and Ortner, 1984). Conversely, as shown dramatically by the recovery of DNA from well-preserved wooly mammoths, there is a corresponding decrease in the degradation of DNA when the temperature is lowered (Handt *et al* 1994). The reaction rate of the degradative processes of hydrolysis and oxidation is halved for every 20°C decrease in temperature below 0 °C (Hoss *et al* 1996). The temperature of a site is affected by a number of variables including altitude (Smith *et al* 2001); as altitude increases, temperature decreases. Skeletons found at high altitude sites, such as mountains (Handt *et al* 1994), are more likely to contain intact DNA than skeletons from lower areas.

Hydrolytic reactions are dependent on the presence of water. Initially the water present in tissue and cells will be sufficient for depurination and breakage of phosphodiester bonds of DNA (Von Endt and Ortner 1984, Lindahl 1993). Environmental water will continue the degradation of DNA. Sources of environmental water include ambient humidity and water content of the soil. Heavy rainfall, flooding and proximity to water sources, such as lakes and rivers, will affect water content of the soil. When water is present in the soil it will participate in hydrolytic reactions resulting in DNA degradation. During heavy rainfall or flood the excess water flowing through the soil and the skeletal material washes the fragments of damaged DNA out of the teeth and bones, in some cases completely stripping them of DNA (Hedges and Millard 1995).

A type of radiation that an organism will encounter during its life is ultra-violet (UV) radiation from the sun; UV radiation will cause dimerization of thymine bases which prevent the accurate replication of the DNA molecule and often lead to apoptosis of the affected cell during life. The DNA damage can be repaired by living cells and apoptotic death prevented. When postmortem tissue is exposed to radiation there are no mechanisms to repair the DNA, but unless the bone and teeth are left exposed on the surface of the soil, the DNA will encounter little UV radiation. A source of ionizing radiation that affects ancient DNA post-mortem is the radioactive decay of minerals in clay based soil (Eglington and Logan 1990). The presence of soil moisture will exacerbate the problem as water will release free radicals in the presence of ionizing radiation (Breimer and Lindahl 1985, Eglinton and Logan 1991, Hutchinson 1985, Lindahl 1993). With no damage repair mechanisms the ionization damage is cumulative and the amount of damage seen increases with increased exposure and time.

The pH of soil, as well as temperature, water content and radioactivity will change the rate of DNA degradation. An acidic soil is associated with an increased rate of hydrolysis of the DNA through depurination (Lindahl and Anderson 1972). In addition to the depurination of DNA when the soil environment is acidic the hydroxyapatite crystals become soluble. The crystals then release DNA and as a result the DNA is no longer protected from degradation. If there is a change in soil conditions and the hydroxyapatite is able to crystallize again, then DNA will bind and once again be protected from degradation. This binding which prevents degradation of DNA will also prevent DNA from being detectable by means of PCR amplification reaction. PCR amplification like DNA degradation, in the form of oxidation and hydrolysis, is an aqueous reaction. Geigl (2002) suggests the use of molecular hybridization instead of PCR amplification in fossil (> 10 000 years old) samples, as the DNA will be more likely to have survived if it is bound to molecules that have prevented aqueous reactions from occurring. Molecular hybridization should be able to identify the presence of DNA without reliance upon PCR amplification.

Also in the soil are microbes - bacteria, viruses, fungi and other microscopic organisms. Temperature affects the metabolic rate and growth of microorganisms in the soil as well as the rates of chemical reactions and there is an increase in microorganism activity in the soil when the temperature is greater than 10°C (Paul and Clark, 1989). Increased microbial activity is detrimental to the structure and preservation of the tooth. Microorganisms degrade the collagen of samples such as the teeth and bones through the release of enzymes and the production of organic acid byproducts (Bar *et al* 1988, Vass *et al* 1992), and then immobilize the constituents by incorporating them into their own structures (Child 1995). Due to the omnipresent nature of microorganisms, they will access the interior of a sample if able. Once the bones and teeth have been accessed, the organic components will be degraded. Therefore, signs of microbial activity indicate that intact DNA is no longer present in a sample. Bones can often be found with microscopic holes in them and there is disagreement about whether this constitutes a sign of microorganism activity (Child 1995). When the soil surrounding the bones was cultured the organisms found were cultured. Then the cultured organisms were tested for their ability to create holes in bone, but there were no microorganisms present that appear to be capable of creating these microscopic holes. Soil bacteria are physically too large to fit into the holes and there was no exonuclease activity detected. The presence of microscopic holes in samples is likely caused by the tendrils of fungi. The fungi decrease the pH in the area around their tendrils and the acidity is destructive to the collagen and DNA.

The environmental factors described here can work to degrade or preserve DNA, however, it is more common for DNA to be degraded than it is for DNA to be preserved intact.

4.8 The Uses of Ancient DNA

Ancient DNA is used to study people, populations, animals and microorganisms. Anything that was part of or in contact with the subject and has been preserved is a potential source of DNA; there has been success extracting DNA from fossilized coprolites, both of humans and sloths, as well as the more commonly used materials of teeth and bones (e.g Poinar *et al* 2003). Before the discovery of high fidelity and relatively easy DNA amplification, other methods were used to study past populations. The oldest methods are the use of metric and non-metric traits to determine genetic distances. An example of a non-metric trait is the presence or absence of Carabelli's cusp in the incisor (Scott 1980), a metric trait is one that is measured such as the length of the crown of the tooth (Moorrees 1957). When compared with modern DNA, information about the origins and evolution of species and populations can be gained.

The species examined most frequently is *Homo sapiens sapiens* (e.g. Kaestle and Smith 2001), but other hominids such as Neanderthals provide valuable information about the human species, and Neanderthal samples have been analyzed when they were available (Krings *et al* 1997).

Ancient DNA when used in conjunction with anthropological and archaeological data can provide insight into cultures and their social practices (e.g. Keyser-Traqui *et al* 2003). One of the uses of ancient DNA is to establish genetic affinities of populations. This is commonly used to establish the origin of a group of people, e.g. the native North Americans (e.g. Starikovskaya *et al* 1998). Prehistoric and modern North American populations have been studied. The pre-historic population of the Great Basin area, in the Southern United States, was examined and it was found that the population structure was consistent with the oral history of the region; that the pre-historic population had moved north and a new population had moved into the area rather than the interbreeding or a slow change in the population (Carlyle *et al* 2000, Kaestle and Smith 2001).

Another aspect is the establishment of relationships between individuals within a population rather then the relationships of populations with each other. The cemetery of Egyin Gol in northern Mongolia was used from third century B.C. to the second century

A.D., but appeared to be a special cemetery due to the small number of burials present and the high degree of relatedness between the individuals buried there. The climate was cold and the bones and archaeological artifacts were very well preserved, so that the samples collected specifically for DNA extraction, with appropriate precautions to prevent contamination, were used successfully by the researchers to determine relationships among the burials in the cemetery. Three distinct aspects of DNA were used to determine different relationships: Autosomal STR's were used to study close parentage relationships, Y-chromosome STR's were used to determine paternal relationship and mtDNA was used to determine maternal relationships. In all cases but one, there was concordance between all three methods of determining relationship, and while there were differences noted between the Egyin Gol and modern Asian mtDNA haplogroups, there were sufficient similarities to allow the assignment of individuals to haplogroups (Keyser-Traqui *et al* 2003).

DNA is also used to authenticate claims of relationships to famous people; a woman who claimed to be the daughter of the German Emperor, Kaiser Wilhelm II, had her claim tested when DNA was removed from a preserved tooth sample (Pfeiffer *et al* 2003). The nine skeletons found in a shallow grave in Ekaterinberg, Russia, were the suspected remains of the Romanov family, the last Russian Czar and his family. Through examination of mtDNA and nuclear STR's it was possible to positively identify the remains as those of the Czar, the Czarina, three of their five children, the royal physician and three servants (Gill *et al* 1994). Even in the relatively short history of the United States the final resting place of Jesse James was determined through comparison of putative remains, teeth and hair, with living descendents (Stone *et al* 2001).

The disease causing organisms of ancient plagues is a topic that has been studied (e.g. [illegible]agrigorakis *et al* 2006). Blood-borne pathogens can be recovered from teeth (Ab[illegible]haram *et al* 2004). This allows researchers to study teeth from putative victims of disease and determine if they were infected with a pathogen. The use of unerupted teeth from juveniles, which were buried in a mass grave, allowed researchers to be positive about which individual the tooth came from and it was thought that the lack of apical foramen helped preserve the DNA from exposure to a degrading environment. However, later studies using adult teeth demonstrated that there was little contamination of the DNA, despite the presence of an open apical foramen. These studies have confirmed the disease causing organism from the Medieval Black Death that was so devastating during the 14th century was *Yersinia pestis* (Raoult *et al* 2000).

Animals such as the declining otter populations (Pertoldi *et al* 2001), the extinct wooly rhinos (Orlando *et al* 2003) and extinct cave bears (Hofreiter *et al* 2004) are some of the species that have been studied. In the case of some animals, such as the otter, DNA analysis is used to determine the degree of variability left in declining populations and the information used in conservation efforts.

4.9 Criteria for Authenticity

Contamination with modern DNA is an issue when working with ancient DNA. The ancient DNA has suffered damage during the post-mortem interval and is fragile. Modern DNA contains no damage and is present in much greater amounts. As a result when the DNA is extracted and amplified the modern DNA is preferentially amplified. In response to this issue there have been recommendations regarding protocols and criteria that prevent contamination and support the authenticity of results (Pääbo *et al.* 2004, Cooper and Poinar 2000).

Pääbo *et al* (2004) and Cooper and Poinar (2000) recommend similar criteria for ensuring the authenticity of DNA. A combination of both Pääbo and Cooper's recommendations has ten criteria that should be considered when working with ancient DNA. A list of the criteria and an explanation of the degree of compliance in this project follows in this thesis work.

1) *The cloning of amplification products and sequencing of the clones.*
2) *Extraction Controls and PCR controls.*
3) *Repeated amplifications.*
4) *Quantification.*
5) *Inverse correlation between amplification efficiency and length of amplification.*
6) *Biochemical assays of macromolecular preservation.*
7) *Exclusion of nuclear insertion of mtDNA.*
8) *Reproduction in a second laboratory.*
9) *Physically Isolated Work Areas.*
10) *Associated Remains.*

4.10 PCR

The use of Polymerase Chain Reaction (PCR) in the field of ancient DNA study has allowed for the study of previously unusable amounts of DNA. The highly sensitive PCR required some adjustments for use with the heavily damaged DNA and the co-extracted PCR inhibitors. The PCR amplification products are designed to have a product length of less than 180 base pairs (bp); it has been shown that 180 bp is the longest that will amplify reliably in ancient samples (Mooder *et al* 2003). The polymerase enzyme Taq has been optimized for use with ancient DNA. Previously hot start PCR was used to keep the Taq separated from the PCR mixture until a high enough temperature was reached, this prevented mispriming of the primers. At lower temperatures the primers are less specific and will bind to sequences that are not a 100% match. If the Taq polymerase is present in the mixture it is possible that there could be amplification of an inappropriate product. Another method that is used to achieve the same effect as hot start PCR is to use "Platinum Taq". The Platinum Taq is attached to an antibody and will not be activated until the antibody is removed when the temperature of 94 °C is reached and held for a minute. Invitrogen Platinum Taq was used for this study. Bovine serum albumin (BSA) is added to many PCR mixtures. The addition of BSA to the PCR mixture will mitigate the effects of PCR inhibitors on the amplification reaction (Al-Soud and Råådström 2000).

4.11 Restriction Fragment Length Polymorphisms

The earliest identifications of haplogroups used the presence and absence of restriction digestion sites (Denaro *et al* 1981) and haplogroups can be easily identified with this method. There are some circumstances where it is still desirable to perform a restriction digestion instead of sequencing a product. Restriction digestion takes less time and costs less then sequencing a PCR product. However, a continual need for amplified product and incomplete restriction digestion combine to make the use of restriction digestion for the identification of haplogroups impractical. PCR amplification and sequencing are commonly used as well. Sequencing requires less amplified sample to determine the haplogroup. There was some restriction digestion analysis used during this study; the reaction conditions that were used are listed below.

4.12 Sequencing

In this study the majority of sequencing that is carried out is direct sequencing. However, three of the samples have been cloned and a sample of the resultant clones has been sequenced. The sequencing of clones has the advantage that it separates all the PCR products allowing the identification of all the sequences that are present in the PCR amplification mixture. The true consensus sequence can then be determined by examining all the species of PCR products that are present. But this very ability to separate out the PCR products is a great disadvantage as well, the sensitivity of cloning means that there is a potential for contamination to occur during the cloning process. Cloning is appropriate for the preservation of the PCR products and the colonies can be stored at -80°C in glycerol. The advantages of direct sequencing are that it can be done quickly and there is little opportunity for additional contamination to occur; while a disadvantage is that the direct sequencing of PCR products will only show the majority of the PCR products. If there is authentic and contaminated sequence in the mixture, the most prevalent species is the one that will be seen, and considering the robust nature of modern DNA when compared to ancient DNA, the sequence seen will be the contaminating sequence. Even if it is present the authentic sequence will be masked.

There is a class of mutations that is seen as a result of the suboptimal conditions in sequencing that occur when working with aDNA, called phantom mutations (Brandstätter *et al* 2005). These phantom mutations will appear most often in the light strand of mtDNA and will be repeated if the sample is sequenced again. To prevent the use of phantom mutations to determine haplogroups, it is necessary to sequence both strands of the mtDNA, the heavy and the light, and then use nucleotides that are present in both strands for assigning the haplogroups. The use of polymorphisms that are present in both the heavy and light strand should allow the identification of apparent polymorphisms that are due to phantom mutations and sample degradation. There are some sites that are more prone to the appearance of phantom mutations than others. Some of these sites that are prone to phantom mutations are also essential to the determination of haplogroups. Any mutations that appear in only one strand or do not occur for all sequencing reactions of the DNA, can be considered phantoms mutations and should be not be used when determining the haplogroup of the sample. The majority of these phantom mutations occur at a small number of sites. Some of the sites that are covered by this study that are prone to phantom mutations are 16241, 16265, 16267, 16270, 16271, 16278, 16283, 16293, 16295, 16296, 16303, 16305, 16311 and 16326 . Sites 16278 and 16311 are prone to phantom mutations and are used for the determination of the haplogroups that were studied.

Chapter 5: Teeth: Growth, Structure and Diagenesis

5.1 Introduction

To gain the most information from a new material, it is necessary that the researcher have an understanding of the processes and factors that are involved in the growth, life and degeneration of the material. Teeth are a relatively new material for the extraction of DNA from ancient samples. It is very useful to understand how teeth grow and change in the body, the materials that teeth are made of and how those materials interact with DNA and their environment. This chapter will review these processes.

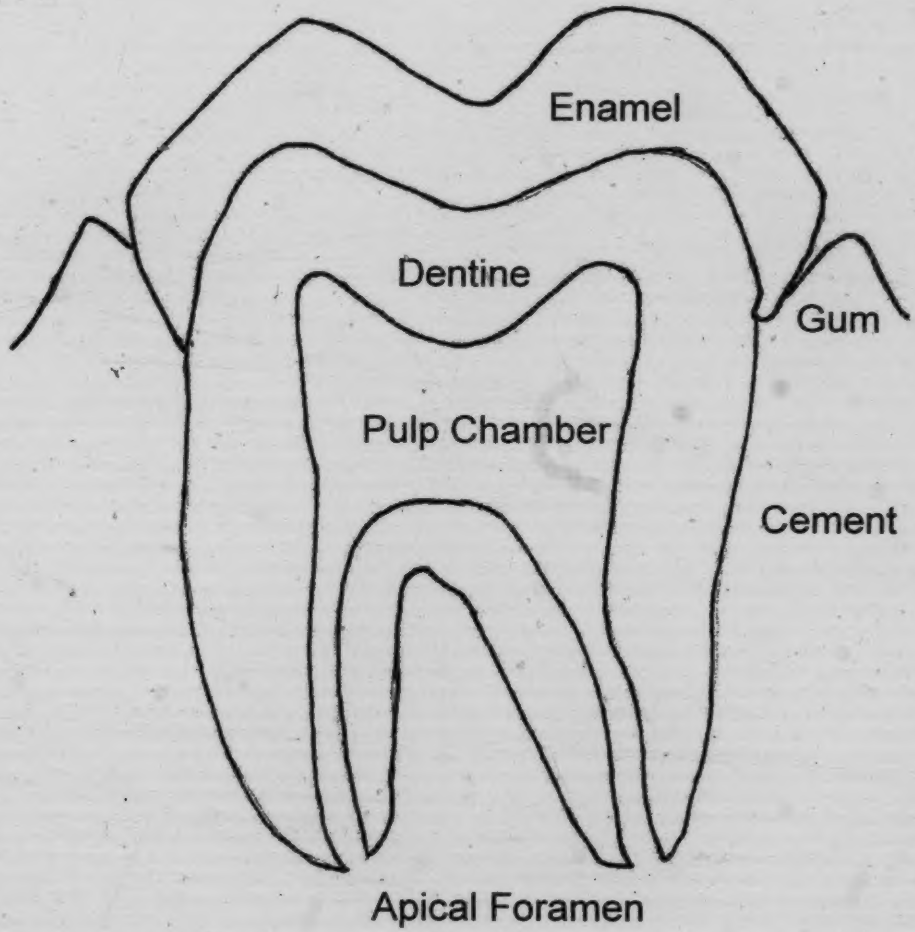


Figure 5.1 – Diagram of Tooth Structure

5.2 The Growth of Teeth

Teeth go through six distinct stages of growth that begin shortly after conception and continue until the late teens. These are the formation of the lamina, the bud stage, the cap stage, the bell stage, crown formation, eruption and for deciduous teeth a seventh stage, exfoliation. Understanding the growth of teeth is important for understanding their structure, biology and chemistry, as well as for the estimation of age.

The development of the teeth begins early in life. Six weeks after conception the mesenchyme condenses into the two arcs of the developing jaw. The arcs of mesenchyme are covered by the epithelial layer of the mouth and the oral epithelium thickens where the teeth will grow, which is now known as dental lamina (Brownell and Slavkin, 1980).

Shortly after the dental lamina is formed the bud stage begins. The newly formed dental lamina penetrates into the mesenchyme to form buds of what will be the teeth, and then the underlying mesenchyme reacts to the lamellar buds by condensing around the buds. By ten weeks after conception all of the buds for the deciduous teeth have developed, but the buds for the permanent teeth start developing at the seventeenth week and the last of the buds do not appear until after birth. The dental lamina that has penetrated into the mesenchyme develops into the enamel producing organ. At this stage there will be ten swellings in each jaw, one for each of the deciduous teeth, with the number of buds increasing as the permanent teeth start to develop. Each tooth will proceed through five stages with the timing varying for each tooth.

Next is the cap stage. The bud now develops a depression within it as the dental lamina begins to wrap around the condensed mesenchyme. The mesenchyme that is now contained within the cap of dental lamina is called the papilla and will later develop into the dentine producing organ. The layer of mesenchyme outside the cap will differentiate into the cement and periodontal ligament. At this stage the tissues for all the major components of the tooth are developed.

The bell stage sees a deepening of the cupped shape of the enamel organ (the dental lamina) and differentiation of the cells of the tooth. The enamel producing cells are epithelial in origin and the cells of the dentine and cement are mesenchymal in origin. This is when the shapes of the future cusps are formed. The cusps are formed by differences in epithelial cell division. While most of the cells continue to divide, some of them stop; this results in a buckling and folding of the enamel organ. This folding provides the pattern of the future crown with the site of the cusps being determined by the cells that stop dividing. The papilla neighbors of the epithelial cells that have stopped dividing differentiate into odontoblasts and begin to secrete predentine. Meanwhile the epithelial cells differentiate into ameloblasts and begin to secrete enamel. The differentiation proceeds in a wave that spreads around the tooth germ. As the first odontoblasts and ameloblasts differentiate they begin to secrete matrix; the predentine matrix and enamel matrices are laid down in layers with the predentine being secreted before each corresponding layer of enamel. The shape of the root is determined by Hertwig's sheath (Webb *et al.* 1996), a finger of the enamel organ that has been extended as a hollow tube. The odontoblasts continue to secrete predentine after the crown is complete until the root has been formed.

The crown of the tooth is formed by the process of secretion. The ameloblasts and odontoblasts secrete layers of enamel and dentine matrix on to each other. The meeting of the dentine and enamel is the enamel-dentine junction. The tips of the cusps, where the epithelial cells stopped dividing, are the first to be secreted. Both dentine and enamel are initially secreted as a mostly organic matrix. The matrices are matured as the organic portion, protein, is removed and replaced with the mineral phase, hydroxyapatite (Simmer and Hu 2001). Maturation is a long term process with the shape and size of the mineral crystals changing. The degree of replacement with minerals varies between the different tissues of the teeth and range from the almost entirely mineral enamel to the cement, which has an organic content that is similar to bone. Once the occlusal surface of the tooth is secreted, the sides of the crown are formed in sleeve-like layers. The layers of enamel at the side of the tooth are thinner than the enamel at the occlusal surface. The end of crown formation is not the end of secretion, the odontoblasts are continuing to secrete pre-matrix, forming the root and canal of the tooth. Secretion ceases when the apex of the root is formed. Once the ameloblasts are finished secreting enamel, they die. Because there are no living cells left in the enamel, it can not be remodeled after growth is finished. Unlike the ameloblasts, the odontoblasts remain alive after their initial secretion is finished; the cell bodies are in the layer of dentine that is immediately adjacent to the pulp cavity. From there the odontoblasts have processes that reach back into the dentine to the enamel-dentine junction. The odontoblasts remain active enough that the secretion of secondary dentine may be sufficient to force them into dormancy as they are cut off from the pulp.

Eruption is the last stage of formation that all of the teeth, both deciduous and permanent, will go through. The completion of the root triggers the eruption of the tooth. This is a long process because the bone of the jaw needs to remodel as the tooth moves through it. The osteoclasts and osteoblasts of the bone work to remodel and form the alveolus of the tooth. The deciduous teeth erupt in the two years after birth; for permanent teeth, eruption occurs approximately three years after the crown has been completed. There are points within the stage of eruption of each tooth that mark the degree of eruption: alveolar emergence is the point when the tip of the tooth reaches the crest of the jaw, gingival emergence is when the tip of the tooth is seen to be appearing through the flesh of the gums. While this can be assessed in the mouths of living patients, once the flesh has been degraded from the jaw it is more difficult to determine the line of the gums and therefore more difficult to determine gingival emergence. After the tooth has erupted it begins to experience wear. This is first seen as the removal of the perikymata, a series of wave like ridges on a portion of the crown and which are indicative of the layering involved in the formation of teeth. The wear on a tooth can become so extreme that the pulp of the root recedes as the enamel is worn away.

For deciduous teeth the last step is exfoliation. Exfoliation is triggered by the formation of the permanent tooth root beneath it. The deciduous tooth root is resorbed and the bone of the jaw remodeled to allow for the loss of the deciduous tooth and the movement of the permanent tooth into place (Sahara and Ozawa 2004). The deciduous tooth acts as a guide to bring the permanent tooth into the correct alignment within the mouth, but if the deciduous tooth has been lost or moved the permanent tooth may grow in crooked. In some people one or two of the deciduous teeth are retained and no permanent tooth grows. This presents a problem because the deciduous teeth are smaller and more prone to wear than their permanent counterparts.

The pattern of growth and eruption of teeth is predictable. Many studies have examined living and deceased children to determine the stage of the dentition at standard ages. The information gathered has been used to develop many different ways of assigning age, each with their own degree of accuracy. However the most common method used is a chart developed by Ubelaker (Ubelaker, 1989). Despite the predictable nature of tooth development, individual variation makes it important that the whole suite of teeth be used to determine age.

5.3 The Structure of Enamel

Dental enamel is the portion of the tooth that is easily seen in the mouth. Enamel is one of the hardest and most enduring components of the body. However, a lifetime of use can wear the enamel away from over 2 mm until the underlying dentine is exposed. The dental enamel has both a 98% mineral and a 2% organic portion. The heavy mineralization of enamel with hydroxyapatite helps prevent the degradation of the teeth (Boyde 1989).

When enamel matrix is first secreted it is one third organic. The mineral portion is apatite crystals which are small at this stage, 30nm, and the orientation of the crystals is dependent on the ameloblasts. Once the ameloblasts have finished secreting the enamel, they change function and begin to break down the organic portion of the enamel. Meanwhile the apatite crystals are growing in size, from 30nm to 50-100 nm (Robinson and Kirkham 1982). The maturation of the apatite crystals and the removal of the organic protein give enamel the familiar properties of hardness and resistance. The resorption of the organic portion of enamel is the last function of ameloblasts before they die.

The ameloblasts are not synchronized in the timing of their maturation and deaths. They differentiate and secrete in zones, with the occlusal surface being the most mature part of the enamel, where the ameloblasts have died. Immediately below this is the maturation zone where the organic matrix is mostly resorbed and the apatite crystals are of intermediate size, but the ameloblasts are still active. The transitional zone is below this and very little resorption has occurred at this point. The last zone is the forming edge, this is where the initial enamel matrix is being laid down in layers. There has not been time for any modification of the enamel to occur by this point.

When examined closely these layers give the enamel three distinct prismatic structures. Pattern 1 enamel has discontinuities between the prisms, pattern 2 has the prisms packed in sheets and pattern 3 has interlocking prisms. The prism patterns are interesting from an evolutionary standpoint. Humans and their close relatives, the gorillas and chimpanzees, have thin layers of prism free enamel and pattern 1 at the surface of the crown. In humans and *Sivapithecus* pattern 3 makes up 90% of the enamel while gorillas and chimpanzees only have 60% pattern 3 enamel. The different patterns do not seem to convey an evolutionary advantage, but rather are indicative of the lineages that have evolved into the separate species (Martin 1986).

One of the visible instances of circadian rhythms in the human body is the striae of Retzius, lines seen in the enamel following the pattern of layering during growth. The striae of Retzius are variable in definition and colour, and represent a 7-10 day cyclical variation in the activity of the ameloblasts (Dean 1987) but the cycle does not appear to start until after birth. The first stria seen in a tooth is the neonatal line and represents the developmental state of the enamel when the individual was born. The neonatal line can be used to differentiate between stillbirths, which will have no neonatal line, and neonatal deaths, which will show the distinctive neonatal line (Schour 1936).

The activity level of the ameloblasts is affected by stress; during periods of severe illness or nutritional deficiency the ameloblasts do not secrete enamel at the regular pace. This leaves a furrow across the surface of the enamel that is often detectable to the naked eye. Called linear enamel hypoplasia, these furrows are very useful for determining the timing and severity of stress in ancient people (Cunha *et al* 2004).

Very little of the enamel is organic, but the majority of that organic material is the protein amelogenin. Amelogenin is coded for by the amelogenin gene that is found on the X and the Y chromosomes. The X chromosome copy has a six base pair deletion, compared to the Y chromosome copy. This size difference is useful in determining the sex of an individual by PCR. A female will have a single large band of 106 bp, while a male will have two distinct bands of 106 bp and 112 bp when the PCR products are visualized on a gel. The 112 bp band represents the Y chromosome and the 106 bp band represents the X chromosome (Akane *et al* 1991). The majority of the amelogenin in enamel is produced from a single X chromosome and the amelogenin gene on the Y chromosome produces very little amelogenin protein. Females have two X chromosomes the second X chromosome is condensed into a Barr body and is inactivated.

5.4 The Structure of Dentine

Dentine comprises the bulk of the root of the tooth. Like enamel, dentine is composed of both an organic phase, collagen, and a mineral phase, hydroxyapatite. The organic content, collagen, of dentine is much higher than that of enamel. The dentine is secreted by odontoblasts which continue to secrete dentine throughout the lifetime of the tooth.

There are five main types of dentine: the circumpulpal dentine comprises the bulk of the dentine in the crown and the roots and immediately surrounds the pulp chamber, mantle dentine is the thin layer of dentine that underlies the enamel-dentine junction (EDJ) and cement-enamel junction (CDJ). Primary dentine is formed during the main growth of the tooth, while secondary dentine is slowly and predictably laid down during the life span of the tooth and can be used in age estimation (Meinl *et al* 2007). Finally tertiary dentine is secreted by the odontoblasts on to the lining of the pulp cavity in response to damage such as inflammation due to infection.

The bodies of the odontoblasts lie near the lining of the pulp cavity and their tubules penetrate deep into the dentine. The tubules are particularly dense when they are aligned in the direction of the enamel-dentine junction. Although after differentiation the odontoblasts cannot divide any more, they do remain viable and capable of secreting dentine for the rest of lives, but if they become too deeply buried in dentine the odontoblasts become dormant.

The dentine matrix is secreted by the odontoblasts as predentine and the odontoblasts are also responsible for the mineralization of the predentine. There are three different types of dentine matrix secreted by odontoblasts: mantle dentine, intertubular dentine and peritubular dentine.

The intertubular dentine is also known as circumpulpar dentine, the most abundant dentine in the tooth. The largest component of the intertubular dentine is collagen fibrils. The fibrils are interspersed with ground substance. Ground substance is mostly water and is present in dentine and cement but not enamel. The orientation of the fibrils is random, but in general they run parallel to the surface of the forming predentine. Where they are very dense the fibrils are organized into bundles. Where the fibrils are sparser some of them will run parallel to the tubules of the odontoblasts (Bosshardt and Schroeder 1992).

The mantle dentine is characterized by the accumulation of fibrils that are both denser and coarser than the fibrils in intertubular dentine. At the EDJ the fibrils are oriented parallel to the tubules of odontoblasts. The sides of the tooth, where the CEJ is, has bundles of fibrils oriented so that they are angled towards the apex of the root.

The peritubular dentine lines the tubules of the odontoblasts and contains very few fibres. The thickness of the peritubular dentine lining is highly variable and is surrounded by intertubular dentine. The peritubular dentine is not found at the surface of the predentine like the intertubular and mantle dentine is; instead peritubular dentine is secreted before the mineralization and maturation of the predentine matrix into dentine rather then being secreted in the initial period (Weber 1968).

After predentine is produced it is mineralized. This step is initiated by the odontoblasts and the mineral phase of dentine manifests as calcoshperites. Odontoblasts initiate crystallites by budding off vesicles that then act as the initiation centres for the calcospherites which are 1-50μm agglomerations. The crystallites that make up the calcoshperites have their long axes radiating out from the centre. These agglomerates of calcoshperites dominate the intertubular dentine. The calcospherites are so dense that they are continuous for most of the root of the tooth, but in poorly mineralized patches throughout the dentine there are spaces between the calcoshperites; the agglomerates in the middle of the dentine are large, while those agglomerates from the edges of the dentine are smaller and spaced further apart (Mishima and Kozawa 1998).

The peripheral dentine at the root and EDJ is poorly mineralized. The poor mineralization gives the dentine a granular appearance. There may be a glass-like heavily mineralized hyaline layer (Furseth 1974).

The spaces between the calcoshperites are referred to the interglobular spaces (Hofman-Axalthelm 1981). Like linear enamel hypoplasia, the interglobular spaces reflect periods of stress during growth. The frequency and prominence of interglobular spaces increases with stresses such as deficiencies in vitamins A or D and the accompanying rickets (Mellanby 1934). This means that it is possible to confirm a case of rickets or match up isolated teeth to a single individual. However because interglobular spaces reflect different stresses with the same response, it is much easier to asses the degree of linear enamel hypoplasia from the exterior of the tooth.

The odontoblasts secrete dentine during the whole life of a tooth. The dentine secreted during growth is primary dentine, while the dentine secreted in response to damage is secondary and tertiary dentine. The amount of secondary dentine secreted in the crown is related to the incidence of caries, while the secondary dentine found in the root is related to age (Mendis and Darling 1979). As a person ages the amount of root secondary dentine increases. Secondary dentine is indistinguishable from primary dentine except for the clear dividing line between the primary and secondary dentine (Vasiliadis *et al* 1983).

Tertiary dentine is secreted in response to stress on the tooth. This includes the stress of caries and the wearing down of the occlusal surface due to a rough diet. The volume of tertiary dentine can be so large that it occupies most of the pulp cavity and root canal. There are very few tubules in tertiary dentine and they are not continuous with the tubules of the primary dentine. The same wear and caries that cause tertiary dentine to be secreted can expose odontoblasts to the oral environment; exposure causes the odontoblast processes in the tubules to die. The empty tubule is sealed with the tertiary dentine and remains full of air (Zerosi 1988).

5.5 The Structure of Cement

Cement is present in small quantities around the exterior of the root, where it serves as an attachment point for the periodontal ligament. The cells that produce cement are the cementoblasts which lie within the periodontal ligament and are fed nutrients by the same vessels that supply the periodontal ligament. Like bone, the cement remodels through out life and not only are the cells active like odontoblasts, but they also actively divide, allowing for the repopulation of cement that has been damaged (Jones 1981). The Hertwigs sheath provides the shape for the growth of cement. On the exterior of Hertwigs sheath the cement is laid down and in the interior the dentine is secreted. The secretion of cement by cementoblasts starts once the odontoblasts begin the formation of the root. Cement does not have any mineralization centres of its own; instead the mineralization spreads from the predentine to the precement. The sharing of the mineralization centres means that the cemento-dentine junction is not sharply defined (Owens 1973).

When a cementoblast is trapped by the secreted precement it becomes a cementocyte. As the cement is being secreted around the cementocytes there is a space trapped around the cells. Known as lacunae these spaces vary in both shape and size. Little processes, called canaliculi, spread from the trapped cementocyte to the surface of the cement. The periodontal ligament provides nourishment to the cementocytes which is carried along the canaliculi. When a cementocyte is buried deeply it is not very active and deepest cells may not be viable (Toda *et al.* 1974).

In the periodontal ligament there are the fibroblast cells that secrete and absorb the collagen fibrils; these cells are very active. At the surface of the cement there are the odontoclasts, which resorb the cement and dentine that is around them.

The mineralized phase of cement is equivalent to that of bone; 24-26%. The rest of the cement is made of the collagen fibrils and ground substance. The mineralized phase can be highly variable due to the lack of endogenous mineralization centres. The apatite crystals that do form are very similar to the crystals seen in bone and they mineralize in the same orientation as the fibrils in the cement. Even when the collagen fibrils of the cement have been lost, the apatite crystals show the direction that the fibrils were orientated in.

There are two types of collagen fibrils seen in cement. Those that penetrate from the exterior of the cement are the extrinsic fibres. Extrinsic fibres originate from the periodontal ligament, with their orientation dictated by the ligament. Intrinsic fibres which are produced by the cementoblasts in the cement and their orientations are parallel to the developing surface of the cement (Bosshardt and Schroeder 1992).

There are several types of cement, classified according to the absence and type of fibrils that exist in the cement. Due to the variability it is often difficult to classify cement as belonging to a single type. The simplest type of cement is afibrillar cement. It has no fibrils and no cells and is composed of only mineralized ground substance. As cement is secreted this is the first layer to be laid down and is just a thin covering. Extrinsic fibre cement has no cells, but it does have tightly packed extrinsic fibres which are most commonly seen in teeth before they have erupted.

Mixed fibre cement has both extrinsic and intrinsic fibres while there may be cementocytes in this type of cement as well. It makes up the bulk of the cement around the root. This cement grows quickest at the apex of the root and at the fork of multi-rooted teeth making the entrapment of cementoblasts more likely. Intrinsic fibre cement also has cementocytes, but no extrinsic fibres.

Within the cement there are layers; these very thin layers are not seen everywhere in cement and it is not known what causes them. The layers may be caused by a seasonal variation because it is possible to count the layers and determine the number of years since the root was formed.

The ability of the cement and periodontal ligament to remodel in response to stress is very useful. This remodeling allows for the success of modern braces in changing the position of teeth and the ability to replant a knocked tooth is dependent on the ability of cementoblasts and fibroblasts to divide and repopulate the damaged area.

5.6 Diagenesis

5.6.1 Enamel Diagenesis

Enamel diagenesis is the changes that occur to the enamel after the initial burial, including chemical, physical and biological changes. The enamel is the part of the tooth most resistant to degradation after burial and may be the only part of a body to survive after death. Since very little of the enamel is organic, the structure of the enamel is not greatly affected by the loss of amelogenin. Despite the longevity of enamel it can still be degraded if the tooth has been buried in an acidic environment (Stead *et al.* 1986).

The structure of the enamel may seem intact but the mineral portion, apatite, does experience diagenetic changes. Apatite is a crystal with the chemical formula $Ca_{10}(PO_4)_6(OH)_2$. The crystals in bone and teeth are often imperfect and can have up to three substitutions in a single molecule. The three sites of substitution are referred to as the A site, B site and the labile site. The labile sites are found in poorly crystallized areas. The negatively charged CO_3^{-2} and F^- are common substitutions in apatite. Carbonate substitutes most often at the B site and fluoride substitutes at the A site. The incorporation of substitutions changes the properties of the apatite crystals. A CO_3^{-2} substitution increases the surface area by reducing the size of the crystals and they change in shape from needles to equi-axed. An F^- substitution has the opposite effect. The apatite crystals increase in size, thereby decreasing the surface area.

The more CO_3^{-2} substitutions there are in the apatite crystal the easier it is to dissolve the apatite. After 24 hours the apatite crystals that have reprecipitated have a marked decrease in the amount of CO_3^{-2}. These redeposited crystals start small but with time grow to be larger than the original apatite. The larger crystals and CO_3^{-2} poor apatite make it possible to determine if the sample had been previously dissolved. (LeGeros 1983)

5.6.2 Collagen Diagenesis

In archaeological situations the dentine often becomes soft and brittle (Beeley and Lunt 1980). The break up of the dentine can lead to fracturing of the overlying enamel. The dentine that is near exposed surfaces, such as the root surface and pulp cavity is the first to suffer from diagenesis. A cause of dentine degradation is the breakdown and loss of the collagen fibrils that are an integral part of the structure.

Collagen is the most abundant protein in vertebrates and is the major stress bearing component of teeth and bones. The protein collagen is made of three amino acids linked together into a protein. The form of the protein is glycine-X-Y. The X and Y amino acids are usually proline and hydroxyprolyl. This is known as Type I collagen. Three strands of Type I collagen twist around each other to form a collagen fibre. Five collagen fibres twist around each other to produce collagen fibrils, The closely packed fibrils are responsible for the high tensile strength of collagen. These fibrils are integral to maintaining the shape and integrity of the dentine (Voet *et al* 1999).

When it is in an aqueous environment collagen can be degraded by hydrolysis. Hydrolysis is a chemical reaction which is catalyzed by the ions present in water, H^+ and OH^-. Even in the most arid desert there is a small amount of water that remains trapped within the sample is sufficient for hydrolysis to occur. As with many other chemical reactions, the rate of hydrolysis increases as the temperature increases (Collins 1995). Once the collagen is hydrolyzed it can be moved out of position in the matrix. Flowing water can easily move the short pieces of collagen from the tooth and into the surrounding soil. When the shape of the collagen matrix begins to dissolve the remaining collagen is more prone to hydrolysis and subsequent removal from the tooth or bone. The areas of dentine that are poorly mineralized are more prone to degradation than the well mineralized dentine. The start of degradation in dentine at the root surface and pulp chamber could be due to the small sparse calcospherites, as well as the exposed position the dentine.

When Collins *et al* (1995) examined the pattern of collagen loss they found it to be more consistent with chemical hydrolysis than the enzymatic reaction of collagenases. There is not enough space in dentine to allow hydrolytic enzymes access to the collagen. A method that allows the monitoring of collagen loss is the measurement of tensile strength.

It would be expected if there was a linear correlation between the age of dentine and the amount of diagenesis. However fossilized teeth are often seen to have better dentine preservation then archaeological teeth. The mineral portion of the tooth has been remineralized and the mineral protects the collagen from degradation. The greater the size of the crystals the less surface area they have. The reduced surface area of the mineral crystals protects them from dissolution. The greater size of crystals after reprecipitation could protect the collagen better than the original crystals. (LeGeros 1983)

Chapter 6: Techniques and Methods: A review of current Techniques for Extracting DNA from Teeth

Due to the degraded nature of ancient DNA, there are many technical difficulties involved in working with it. Such problems include: scarcity of samples, post-mortem changes to the DNA, co-extraction of PCR inhibitors and low copy number. There are two main methods for the extraction of DNA from teeth. One requires that the whole tooth is crushed and the resultant powder used for the extraction of DNA, while another requires removal of the interior of the tooth from which DNA is extracted. Both methods retain the possibility of introducing contamination into the DNA extract, therefore much of the focus has been upon the reduction of contamination. The techniques that are optimized for use with ancient DNA are also ideal for use in forensic circumstances.

The greatest amount of DNA is recovered from the extraction of the main body of the root; the amount of DNA is a thousand times greater than that recovered in other portions of the tooth (Gaytemenh and Sweet 2003). Within the root of the tooth is the dentine, a cell rich tissue that is the main source of DNA. Covering the dentine of the interior is the crown of the tooth; the crown is important because it shows the effect of the person's diet and isotopes isolated from the crown can show where a person was born, grew up and lived their life. Preserving the crown of the tooth increases the amount of information that can be gained from a single tooth. Following is a review of a variety of protocols with a short acknowledgement of their advantages and disadvantages.

One of the most efficient methods to access the DNA in a tooth is to grind the whole tooth and extract DNA from the resultant powder. The cryogenic grinding of teeth was reported by Sweet and Hildebrand in 1998. A limitation of this method is the total destruction of the tooth and that the exterior of the tooth is indiscriminately mixed with the interior. The exterior has a greater possibility of being contaminated with modern

DNA than the cell rich interior. When the exterior is included in the materials to be extracted it greatly increases the chances that the DNA extraction will be contaminated.

Bendetto *et al* (2000) used a method that involved the grinding of teeth to recover DNA from human samples retrieved from the Alps. In this case the crown and root of the tooth were separated by a drill saw; the surface of the root was then removed and the whole root was powdered in a grinding mill. This method isolates the cell rich dentine and should extract a large portion of the DNA that exists in the tooth with a minimum of contaminants. Unfortunately, there is complete destruction of the root of the tooth, leaving only the crown of the tooth intact.

Several protocols that aim to remove only the uncontaminated pulp of the teeth have been published. One method has been to split the tooth in half and scoop the pulp from within the interior of the tooth (Grimoud *et al* 2002). The largest problem with this method is that the morphology of the tooth is destroyed during the act of splitting; otherwise the method works well to keep contamination from the exterior of the tooth to a minimum.

The next exploration was to drill through the apical foramen of the tooth and remove the pulp without disturbing the exterior in any way; this has been referred to as a reverse root canal. Cobb (2002) reported success with human samples excavated from a Shang Dynasty cemetery in China that was used from 1300 to 1045 BC. The amount of pulp material that can be removed without disturbing the morphology of the tooth is small and when working with ancient material it is important that the maximum amount of DNA is extracted from the material. This method does not remove enough material to enable the extraction of a significant amount of DNA in many of the ancient specimens.

The samples that were worked with were from 700 to a 1000 YBP, which may explain why Cobb was able to experience success amplifying DNA with a minimum of dentine powder to work with. Cobb did report that there was minimal disturbance of the dentine, leaving sufficient sample for later researchers to extract.

A method with minimal damage to the sample was introduced by Rohland *et al* in 2004. This group did not drill into the tooth, thereby avoiding damage to the tooth. Instead the teeth are soaked in buffers, with the most efficient buffer that they tested containing guanidinium thiocyanate (GuSCN), a chaotropic salt that that will cause DNA to bind to silica particles. After soaking in the buffer for both two days and seven days a strong PCR product was produced. While this extraction method preserves the tooth, this protocol also has the highest risk of extracting contaminating modern DNA along with any endogenous DNA, because any contaminating DNA on or near the surface of the tooth would be eluted first into the solution.

At the Ancient Biomolecules Centre in Oxford England, Gilbert *et al* (2004) evaluated a method to reduce contamination. First, the tooth is encased in silicone, then the silicone is removed from around the root until just the tip of the root is exposed. The exposed root tip is then removed and the dental pulp is removed by drilling the dentine out of the root tip. This method works well to reduce the amount of possible contamination from the exterior of the tooth and the morphology of the tooth is well preserved. Unfortunately, the silicone remains, encasing the remainder of the tooth and the samples are no longer available for any further studies.

Another protocol that was described in 2004 was reported by Shiroma *et al* (2004). The crown of the tooth is removed from the root exposing the dentine in both the root and the crown. The interior dentine of both the crown and the root are then removed and when the dentine removal is complete the tooth can be reassembled with wax; when skillfully done it is not immediately apparent that the tooth had been cut in half. This method works well to extract a large portion of the dentine, while allowing reassembly of the tooth. This method is appropriate for forensic cases where the tooth will be returned to the victim's family and appearance is essential. Reassembly of the tooth with wax contaminates the sample with wax and makes the tooth unsuitable for any further chemical analyses.

The method that is used for this study is a combination and extension of the methods mentioned above. The tooth is held securely in Tygon® vacuum tubing, two thirds of the root is removed and then the interior of the dentine is removed from the crown and remaining one third of the roots. There is no contact of the exterior of the tooth with the interior, minimizing the risk of contamination. A portion of the root is left intact, allowing other researchers to perform further studies. As most samples that undergo this procedure are destined for further analysis the tooth is not reassembled.

Chapter 7: Materials and Methods

7.1 Sample Sites

Modern Teeth: Modern teeth were donated by Dr.Yu from the Department of Medicine and Dentistry at the University of Alberta, N= 48.

Iberian Peninsula: The 20 sample teeth were collected from 8 sites in Spain and Portugal.

Arruda: an open-air Mesolithic shell midden along the Muge River in central Portugal, 7224-7783 YBP, N = 2.

Furninha: a Neolithic cave on the Estremadura coast of Portugal, 5500-6900 YBP, N = 4.

Fontainhas: a Neolithic limestone cave used as a dedicated ossuary, in the Estremadura region of Portugal, 4724 YBP, N = 2.

Casa da Moura: a Neolithic limestone cave used as a dedicated ossuary, in the Estremadura region of Portugal, 5595-6869 YBP, N = 4.

Feteira: a Neolithic limestone cave used as a dedicated ossuary, in the Estremadura region of Portugal, 4660-5297 YBP, N = 2.

La Garma: a cave in Cantabrian Spain with both Mesolithic and Neolithic remains, N = 4.

El Cotero de la Mina: an open-air Neolithic site in Cantabrian Spain, N = 1.

Melides: a Neolithic cave in southern Portugal, 4989-6144 YBP, N = 1.

Khuzhir-Nuge XIV: An Eastern Siberian Cemetery on the shore of Lake Baikal.

There were 34 teeth sampled from Khuzhir-Nuge XIV, a community grave yard from the middle Holocene, 3000 YBP. The climate of the area is very much like

Edmonton, Canada, and stays cool enough to slow the degradation of DNA. The teeth analyzed in this study were collected from the cemetery from 1997 until 2003.

7.2 Tooth Selection

The selection of teeth for extraction of DNA can affect the success of the extraction. The use of molars allows a larger volume of dentine for extraction than the mining of single rooted teeth. However, the state of preservation of the tooth is more important than the volume of the tooth.

The multi-rooted molar teeth are more useful in DNA extraction than the single rooted teeth. There is a larger volume of dentine in molars than there is in single rooted teeth. The larger size also makes molars easier to work with during the mining process, it is easier to hold the tooth and there is more room to work with the drill.

There are three different types of molars, the first molar, second molar and the third molar. The molars erupt in this order. Because the first molar has erupted before the other two molars it has the most wear. The wear on the first molar increases the amount of sclerotic dentine and provides information about the diet. The third molar does not always erupt and is variable in size and shape, making the third molar less desirable for analysis than the second molar. The second molar has less wear than the first molar and is less variable than the third molar. An adult second molar is the preferred tooth for analysis, because unlike a juvenile tooth, the root has not been resorbed.

7.3 New Method for Mining Teeth

The methods discussed in Chapter 6 for the removal of DNA from teeth are destructive and prone to contamination with modern DNA. In response to this problem a new method of DNA extraction from teeth was developed using the information from previously published methods and in consultation with the Department of Dentistry at the University of Alberta. There is minimal destruction of the tooth and contamination is minimized by avoiding the use of material that has been in contact with the exterior, possibly contaminated, surface of the tooth. The procedure for the new tooth mining method is described below.

7.4 Tooth Mining Procedure

1. The teeth to be prepared are chosen. The tooth is then cleaned with 4% w/v hypochlorite and scrubbed with a toothbrush to oxidize and remove any modern DNA that is potentially present on the exterior of the tooth. When clean, rinse the tooth with autoclaved distilled water to remove the hypochlorite and prevent the introduction of a strong oxidizer into the interior of the tooth. Allow to dry while preparing the rotary tool
2. Secure the tooth for mining. Place the crown of the tooth into a piece of sterile Tygon® vacuum tubing and secure the tubing in a conical tube. See figure 7.1.
3. Remove 1/3 of the tooth's roots with a Dremel® diamond cutting disc and a Dremel® rotary tool, exposing the root canal of the tooth, leaving 2/3 of the root attached to the crown of the tooth. Retain the root and place in a labeled sterile container for later use by other researchers. For example, a thin section of the

root, when examined under microscope, can provide information about both the state of preservation of the sample and the age of the individual. See figure 7.2.

4. Change the bit on the rotary tool from the diamond cutting disc to a 1/8" diamond drill from Dremel®. Use the drill to remove the dentine without touching the exterior of the tooth. See figure 7.3. Drilling into the root canal removes the dentine from the interior of the tooth. Stop frequently to remove the resultant powder from the tooth cavity. Any powder that spills or is aerosolized should be treated as contaminated and disposed of.

Figure 7.2 – Tooth root being cut off with a Dremel® tool. This picture illustrates the technique. All work should be done in a dedicated laboratory.

Figure 7.1 – Tooth held in Tygon® vacuum tubing.

Figure 7.3 – Dentine being removed from tooth. This picture shows how the technique should be performed. When processing ancient samples the work should be carried out in a clean environment.

This new method of mining teeth for DNA was tested and compared to the more conventional method of grinding the entire tooth. The results of this testing are explained in Chapter 8.

7.5 Criteria for Authenticity

The criteria for authenticity suggested by Pääbo *et al* (2004) and Cooper and Poinar (2000) are important to the confidence that results from ancient samples are from endogenous DNA rather then contaminating DNA. Despite the importance often only a subset of criteria can be met. In this study the criteria that were met are listed below.

The cloning of amplification products and sequencing of the clones. There was cloning of three of the five samples from Khuzhir-Nuge in Eastern Siberia. There were multiple clones sequenced from each of the amplified samples.

Extraction controls and PCR controls. There were extraction controls that were treated as samples during extraction and then checked for contamination. Both positive and negative PCR controls were used with all amplifications, the positive control showed that the PCR amplification was successful and the negative control demonstrated that there was no contamination of the PCR reagents.

Exclusion of nuclear insertion of mtDNA. The portion of the hyper-variable region that was amplified has not been detected as a nuclear genome insert so this criteria was not a concern in this study.

Repeated Amplifications. A sample was not considered for further analysis until there had been successful amplification from both the A and the B extracts of a sample. Both the extraction and the amplification had to be temporally separate for the A and B samples.

Physically isolated work area. Two physically isolated rooms were used as described in the criteria for authenticity in Chapter 4.

Some of the criteria for authenticity were not followed as closely. The criteria not followed and the reasons why they were not followed are listed below.

The cloning of amplification products and sequencing of the clones. Five of the eight samples from Eastern Siberia and the samples from the Iberian Peninsula were not cloned. Cloning and the sequencing of multiple clones is an expensive and time consuming process, additionally the information that can be gained from direct sequencing is valuable. There was a decision to clone only a few samples for this study and use direct sequencing for all other samples.

Quantification. There was no formal quantification of PCR amplification products during this study. The manual observation of amplification band intensity was performed for all amplifications. All amplifications showed an intensity that was less then the positive control.

Inverse correlation between amplification efficiency and length of amplification. There was a single length of amplification product used in this study, 176 bp.

Biochemical assays of macromolecular preservation. The correlation of the degradation of collagen and the degradation of DNA allowed a visual assesment of preservation. With only three teeth available from each individual from the cemetery of Khuzhir-Nuge and a single tooth available from the sites on the Iberian Peninsula there was not enough sample to spare for biochemical assays.

Reproduction in a second laboratory. There is no readily accessible laboratories close to the University of Alberta campus that are set up to work with ancient DNA. There were limited samples available, three teeth from the Khuzhir-Nuge individuals and a single tooth from the Iberian Peninsula individuals. Reproduction of results in a second laboratory was an impractical step in this study.

Associated faunal remains. Faunal remains found were used for other analyses.

7.6 DNA Extraction

The technique used to extract the DNA from the samples was slightly modified from the method of Boom *et al* (1990). The method used was

1. Mix sample and extraction buffer together.
2. Add silica and incubate.
3. Wash twice with wash buffer.

4. Wash twice with 70% Ethanol.

5. Wash once with acetone and dry.

6. Elute into buffer.

The modifications to this method are listed below.

1. The powdered teeth are left in the extraction buffer overnight, a minimum of 16 hours, at 65°C.

2. The silica binding step was extended to two hours.

3. No changes.

4. No changes.

5. No changes.

6. The final elution step used 80 -100 µl of sterile MilliQ water and is incubated for one hour.

All the extractions were carried out in temporally separated duplicates, different portions from the same sample were extracted at different times, and included extraction blanks that were extracted in tandem, these samples are labeled as A and B and referred to as such throughout the rest of the study.

7.7 PCR

The same primers and PCR programs were used for both the modern and ancient DNA extracts. The amount of DNA extract in the PCR mix when amplifying modern DNA was 0.5 µl and for ancient DNA extracts, 8 µl of template were used.

Two primers were used to amplify a region of the human mitochondrial Hyper Variable Region I (HVI): Primer H 16346 GGG ACG AGA AGG GAT TTT GAC and

Primer L 16211 CCC ATG CTT ACA AGC AAG TA (Mooder 2004). An annealing temperature of 53 °C for 50 cycles was used.

The primers used to amplify for the haplogroup H analysis were: Primer H1 ATA GGA CAT AGT GGA AGT GGG C and Primer H2 GAT TCA TCT TTC TTT TCA CCG (Private communication with Dr. Mooder). An annealing temperature of 58°C was used for the 41 cycle program.

The primers used to amplify for the haplogroup V analysis were: Primer V1 ATG GCA GCT TCT GTG GAA CG and Primer V2 GCC CAA CCC GTC ATC TAC TCT A (Private communication with Dr. Mooder). An annealing temperature of 55°C was used for the 41 cycle program.

The PCRs were performed in 50 µl volumes with Platinum *Taq* (Invitrogen), and 8 µl of the DNA extracts were used for amplification. The PCR mix comprised 1.25 U of Platinum *Taq* in Invitrogen PCR buffer, 1.5mM $MgCl_2$, 1 mM Bovine Serum Albumin, 200µM of each dNTP and 0.8 µM of each oligonucleotide primer. The PCR was performed in a PTC 125 MiniCycler (MJ Research, Boston, MA). The program and PCR cycle were optimized by Dr. Karen Mooder as a part of her PhD work in 2004 (Mooder 2004).

For the amplification of the amelogenin gene to aloow determination of sex there were two primers used: Amel 1 CCC TGG GCT CTG TAA AGA ATA GTG and Amel 2 ATC AGA GCT TAA ACT GGG AAG CTG (Mannucci *et al* 1994). An annealing temperature of 58°C was used for the 65 cycle program.

The amelogenin amplifications were carried out in 50 µl volumes, using 8 µl of ancient DNA extract as the template. The reaction mixture was comprised of 1.5 U of

Platinum *Taq* in Invitrogen PCR buffer, 1.5 mM $MgCl_2$, 0.8 mM Bovine Serum Albumin (BSA), 100μM of each dNTP, 1.6 μM of primer Amel 1 and 2.8 μM of primer Amel 2.

The negative controls used were 42 μl volumes of the PCR Master Mix without any additions. The positive controls were the 42 μl volume of Master Mix with the addition of 1 μl of modern extracted human DNA of known gender and mtDNA haplogroup. The PCR products were analyzed on a 10% polyacrylamide gel and visualized with EtBr. The picture was captured with a Kodak DC 290 Zoom digital camera on a Fisher Scientific UV light box. The files were saved in two separate electronic locations and a hard copy was labeled and placed in a laboratory note book.

7.8 Restriction Digestion

The restriction analysis for haplogroup V was performed in 35 μl reactions: 25 μl of the PCR product was added to the appropriate buffer, 100μg/μl BSA and 0.5 U of the restriction enzyme *NlaIII*. The samples were incubated for 3 hours at 37°C. Haplogroup V was indicated by the loss of the *NlaIII* restriction site at 4577.

The restriction analysis for haplogroup H was performed in a 35μl reaction: 25μl of the PCR product were added to the buffer for *AluI*, and 0.5 U of *AluI*. The reaction was incubated at 37°C for 3 hours. A loss of the restriction site indicated haplogroup H.

7.9 Cloning

Cloning was carried out by a laboratory technician using a PCR-Script cloning kit (Statagene, USA).

7.10 Sequencing

The samples amplified in duplicate were sent to The Amplification Genomics Centre (TAGC) sequencing centre, Department of Medical Genetics (8th floor Medical Sciences Building, University of Alberta). There is the option of direct sequencing from the PCR product or sequencing clones.

1-3 ng of DNA were used for the direct sequencing on an Avant 3130 genetic analyzer. A single sequencing reaction for each forward and reverse strand was preformed. The resultant information was analyzed on Sequence Scanner v1.0 (available for free online from Applied Biosystems). This is a program with many useful features, but it does not allow an overview of the entire sequence on one page. The sequences presented in the appendix of this thesis were created using FinchTV, this free program is available at www.geospiza.com/FinchTV.

7.11 HVI Sequence Analysis

The corrected DNA sequences were compared with the Cambridge Reference Sequence (Anderson *et. al.* 1981) using the BLAST 2 Sequences feature on the NCBI website (http://www.ncbi.nih.gov). To determine haplogroups the sequences were aligned and a consensus between the forward and reverse reactions was determined. The consensus was then compared to reported haplogroups from Derenko *et al* (2003) and Pereira *et al* (2000), and a haplogroup was assigned.

7.12 DNA Damage

For the purposes of this study DNA damage was defined as polymorphisms that were not seen consistently between the A and B sequencings of the samples. Additionally if the polymorphism was not seen in both the heavy and light strands of the A and the B samples then it was regarded as damage.

7.13 Materials

Three groups of teeth were worked with in this study: modern teeth, teeth from the Iberia Peninsula and teeth from Eastern Siberia. The modern teeth were used in the development of the mining method. These teeth had undergone various treatments, such as bleaching and X-ray imaging, which are common when dealing with teeth. The second group is a set of teeth that were interred 7000 to 10000 YPB, which were recovered from sites in Spain and Portugal, the Iberian Peninsula. These teeth come from a time when the populations in that area were transitioning from a hunter-gatherer lifestyle to a pastoral lifestyle. Genetic information from these teeth could help determine the mode of transition that occurred in the area. The third group of teeth was from a 3000 YBP cemetery of KNXIV in eastern Siberia. This culture remained hunter-gatherers until relatively recently; there has been a succession of cultures in the area with one long hiatus period during which the area had a small population which left few artifacts. Information from these teeth will be used to establish biological relationships between individuals at the cemetery and between different cemeteries.

7.14 Comparison of Mining and Grinding of Teeth

The newly developed mining method was compared to the established grinding protocol; to compare the two different methods of obtaining DNA the amplification strength of the PCR products were compared. The observations made to score the amplification strength were subjective To try to ameliorate the issue of being subjective, a second researcher was asked to evaluate the data. Due to insufficient quality of data, the teeth that had been bleached were not evaluated in this second round of scoring.

Chapter 8: Results

8.1 Modern Teeth

The modern teeth used for this experiment were obtained from dentists and had been extracted during regular dental procedures; the teeth were stored and treated in a heterogeneous manner. The conditions that the teeth were subjected to were noted.

8.2 Comparison of Mining and Grinding Methods in Modern Teeth

The modern teeth used to test the mining method had been exposed to different conditions and had other factors that potentially affected the ability to recover DNA from the teeth. Some of the teeth had not been exposed to any of the conditions and did not have any factors that could affect the retrieval of DNA. With nothing done to them these teeth were used as a comparison to the teeth that had factors affecting the DNA. Each of the factors observed are those that could be found in a sample (e.g. caries) or might be done to a sample during cleaning and examination (e.g. x-ray radiation exposure, hypochlorite exposure). Even the examination of teeth that had a root canal performed simulated an attempt to extract DNA from a tooth that had previously been mined. The comparison of DNA extracted after the tooth is ground or mined tests the value of the new mining technique.

The factors tested in comparisons of modern teeth were:

- Evidence of a root canal, with the root mainly intact
- X-ray radiation exposure
- Presence of cavities, filled and unfilled
- Long term exposure to hypochlorite (3 months in a 6% w/v hypochlorite solution).

There were sufficient samples worked with to allow the use of parametric statistics. Initially the only factor analyzed was the presence or absence of a visible amplification product. This method of scoring produced no significant difference between any of the conditions that the teeth had been subjected to. In order to quantify an observable difference the amplifications were then scored on a different criteria. The absence of a product was scored as a 0, a strong repeatable product was scored with a 3, and the scores of 1 and 2 were assigned to products that did not amplify strongly all the time.

Each of these conditions that potentially affected DNA were compared to teeth that were unaffected by any of the factors; both mined and ground teeth were included when comparing these factors. The comparison of ground and mined teeth included all the modern teeth examined in this study regardless of the other factors affecting them, to allow for a larger sample size and increased significance for the results. There were one to three amplifications available for evaluation from each of the teeth. T-tests were used to compare the factors being studied, with the degrees of freedom varying but a 95% confidence ($\alpha = 0.05$) being used for each t-test. The results of the t-tests are shown in tables 8.1 and table 8.2. The null hypothesis for these T-tests is that there is no difference in amplification strengths between the treatments being compared. The T-test shows that there is a difference, not if one is better than the other. However, observation implies that the teeth with no exposure to bleach or x-ray radiation have more robust DNA than the treated teeth.

The most important comparison was between the amplification strength of mitochondrial DNA from teeth that had been mined and teeth that had been ground. This was the test that determined if the new mining method was viable as a protocol for the extraction of DNA from teeth. Both researchers who subjectively scored the amplification strengths agreed that there was no significant difference in mtDNA amplification strength between teeth that had been mined and teeth that had been ground. The use of mining to extract DNA from teeth provided as strong DNA amplification as DNA extraction after grinding the teeth.

For mtDNA amplification the two researchers agreed that there was no significant difference between the amplification strength of teeth that had a filling and teeth that did not. There was disagreement about the other factors, root canal and x-ray exposure, so there are no conclusions that can be drawn about a root canal and X-ray exposure affecting mtDNA amplification. One researcher assessed the mtDNA amplification strength of teeth that had been left in 30% [illegible]/v bleach for 62 days. The second researcher was unable to provide an opinion as the data was not preserved due to a problem with the gel scanning system. It was found that there was a significant difference in amplification strength, with a greater amplification from teeth that had not been in bleach.

The evaluation of nuclear DNA amplification strength had the two researchers agree that there was a significant difference in amplification strength between teeth that had been exposed to X-ray radiation or had a filling and teeth that had neither of these factors. The teeth that did not have a cavity or had not been exposed to x-ray radiation had better amplification than the teeth that had cavities and x-ray exposure. The researchers also agreed that there was no significant difference in nuclear DNA amplification strength whether there had been a root canal performed or not. There was disagreement about the potential significance of amplifying nuclear DNA after some teeth had been mined and some teeth had been ground. There was a significant difference in amplification strength between teeth left in bleach and teeth that were not, as observed by one researcher. There was concordance between the two separate evaluations on the significance of a factor in the recovery of DNA in one of three factors for the mitochondrial DNA and two of three factors for nuclear DNA.

Table 8.1 Summary of statistics, factors affecting retrieval of DNA. Nothing done indicates that the tooth had not had a root canal done, had not been x-rayed, did not have a filled caries and had not been submersed in bleach. D.f. = N-1. Total N = 48.

T-Tests

mtDNA Amplification Strength

Factors	t-value	d.f.	Significance
Root Canal v.s. No Root Canal	0.510	30	0.480
X-ray v.s. No X-Ray	4.483	32	0.042
Filling v.s. No Filing	0.000	25	0.987
Bleach v.s. No Bleach	6.333	29	0.018
Mined v.s. Ground	0.684	47	0.413

Nuclear Amplification Strength

Factors	t-value	d.f.	Significance
Root Canal v.s. No Root Canal	2.979	30	0.095
X-ray v.s. No X-Ray	7.471	32	0.010
Filling v.s. No Filling	4.548	25	0.043
Bleach v.s. No Bleach	29.911	29	0.000
Mined v.s. Ground	4.762	47	0.034

The H_0 is that there is no difference between the amplification strengths of the groups of teeth that have been treated differently. The $\alpha = 0.05$.

Table 8.2 Summary of statistics, factors affecting retrieval of DNA re-evaluated by a second researcher. D.f. = N-1. For mtDNA N=41. For Nuclear DNA N= 33.

T-Tests

mtDNA Amplification Strength

Factors	t-value	d.f.	Significance
Root Canal v.s. No Root Canal	8.502	29	Yes, 0.007
X-ray v.s. No X-Ray	1.191	31	No, 0.284
Filling v.s. No Filling	0.833	24	No, 0.370
Bleach v.s. No Bleach	N/A	N/A	N/A
Mined v.s. Ground	0.342	40	No, 0.562

Nuclear Amplification Strength

Factors	t-value	d.f.	Significance
Root Canal v.s. No Root Canal	3.678	23	No, 0.068
X-ray v.s. No X-Ray	13.688	23	Yes, 0.001
Filling v.s. No Filling	4.468	18	Yes, 0.049
Bleach v.s. No Bleach	N/A	N/A	N/A
Mined v.s. Ground	0.111	32	No, 0.741

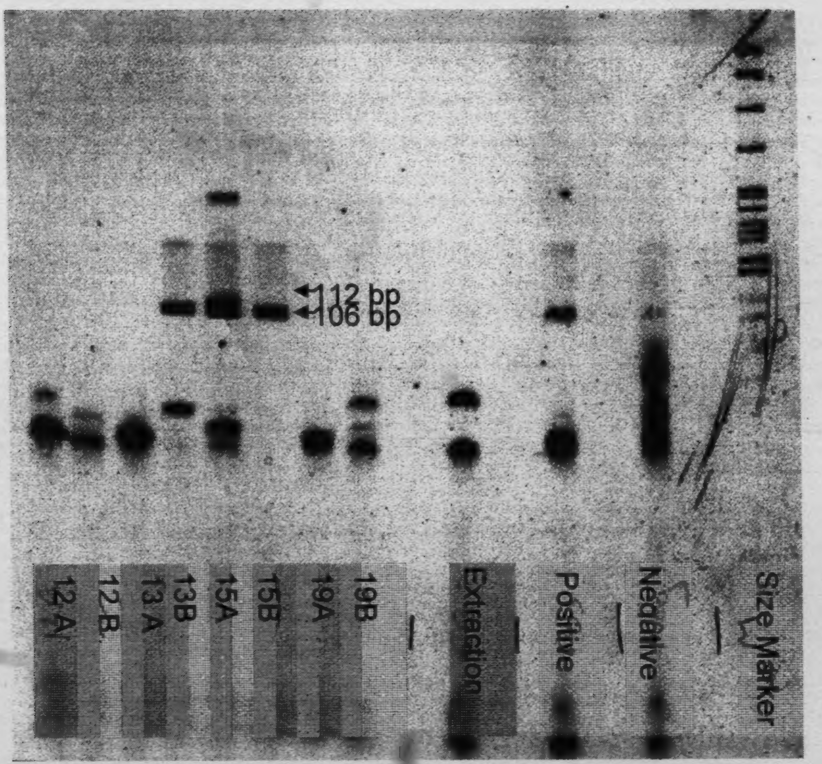


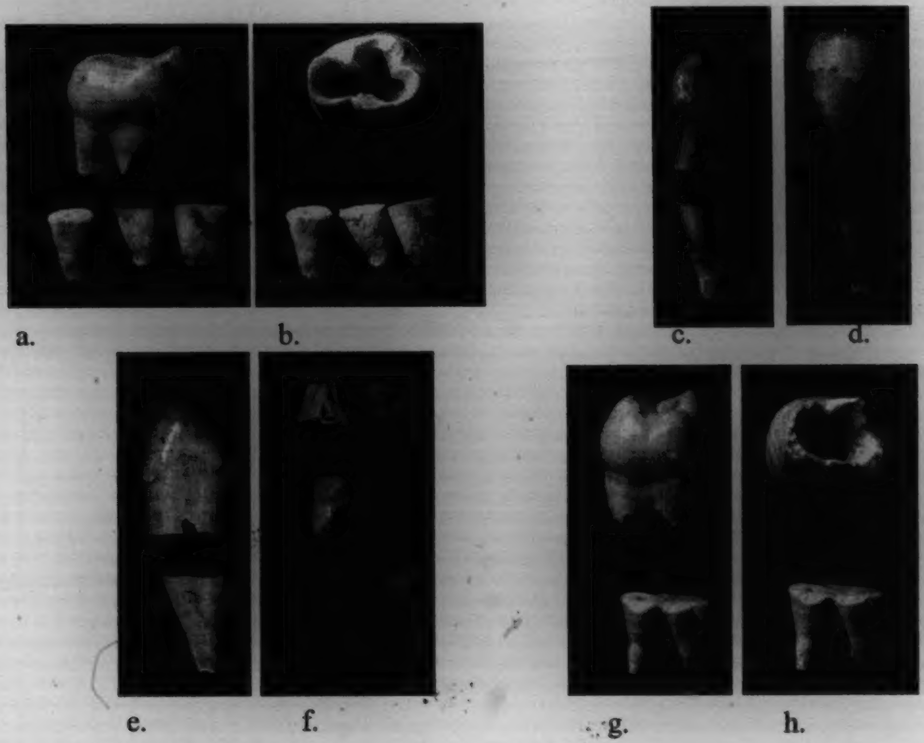
Figure 8.1 - DNA PCR products on a 10% polyacrylamide gel; amelogenin amplification of modern teeth. Sample 12 had a filling and exposure to X-ray radiation. Sample 13, 15 and 19 had nothing done to them. The bands visible at the bottom of each lane are less then 67 bp and are primer-dimers. (Note: there is contamination in the negative lane, but the samples were not used in the final analysis.)

The polyacrylamide gel shown in figure 8.1 illustrates that the method developed for the mining of teeth can be used for the recovery of DNA without contamination. This figure shows PCR fragments amplified from the amelogenin gene on the sex chromosomes. There is a 6 base pair deletion in the amelogenin gene on the X-chromosome when compared to the Y-chromosome. With these primers there is a single band of 106 base pairs if the sample is from a female and if the sample is from a male there are two bands, one at 106 base pairs and the other at 112 base pairs (Mannucci *et al*

1994). The positive control is a female. In figure 8.1 sample 13 B appears to be female, sample 15 A is male since there are two distinct bands; sample 15 B has a single band. When dealing with alleles the phenomenon known as allelic dropout can occur; due to the random nature of the initial amplification step of the PCR and the greater ease of amplifying smaller fragments, the smaller allele has been preferentially amplified over the larger allele, in this case the 112bp allele is not seen (Mannucci *et al* 1994). While it is possible to lose an allele during amplification it is not possible to gain one; therefore, even though there is no 112 base pair band in sample 15 B, the individual that contributed sample 15 is male. The individual that donated the positive control appears to be a female. The laboratory personnel at the HILA, Human Identification Laboratory for Archaeology, were exclusively female at the time of these experiments. The detection of a male sample indicates that it is possible to use the tooth mining method described in this chapter without contamination. Since it is very improbable that the discovery of a male sample could have resulted from contamination; the mining procedure can produce uncontaminated results.

The mining method leaves the majority of the root and the crown intact; however, depending on the preservation state of the tooth there may be damage from handling the teeth. The better the preservation of the tooth, the easier it is for the tooth to survive the mining protocol intact and the more likely that there is authentic DNA present. A tooth with well preserved collagen is firm to the touch, while a tooth with degraded collagen will crumble. DNA and collagen are degraded by many of the same processes; if the structure of the tooth is well preserved it is very likely that the DNA is well preserved as well. When the teeth are well preserved, as at the Iberian Peninsula, there is little to no damage to the tooth during mining (see figure 8.2) During mining of the Khuzhir-Nuge XIV teeth there was a great deal of damage observed to the teeth as they were processed.

Figure 8.2: Iberian Peninsula tooth samples after mining.



8.3 Iberian Peninsula:

These samples had significance for providing potential information on the replacement of hunting and gathering with pastoralism during the Neolithic period. Though the sample size is small and obtained from several different sites, the teeth were used to demonstrate that the mining technique is viable in ancient teeth. If the mining technique is viable and teeth from the Spanish and Portuguese sites contain amplifiable DNA, then the mining method can be used on other teeth and further work can be done in the area with more tooth samples.

Eighteen of the twenty teeth contained amplifiable DNA, of those eighteen, twelve produced amplification products in duplicate samples (Table 1) a success rate of 60%. The samples that amplified were 9.2, 9.3, 9.4, 9.9, 9.11, 9.13, 9.15, 9.16, 9.17, GAA 258, GAA 904 and GAC 421 (Figure 8.3).

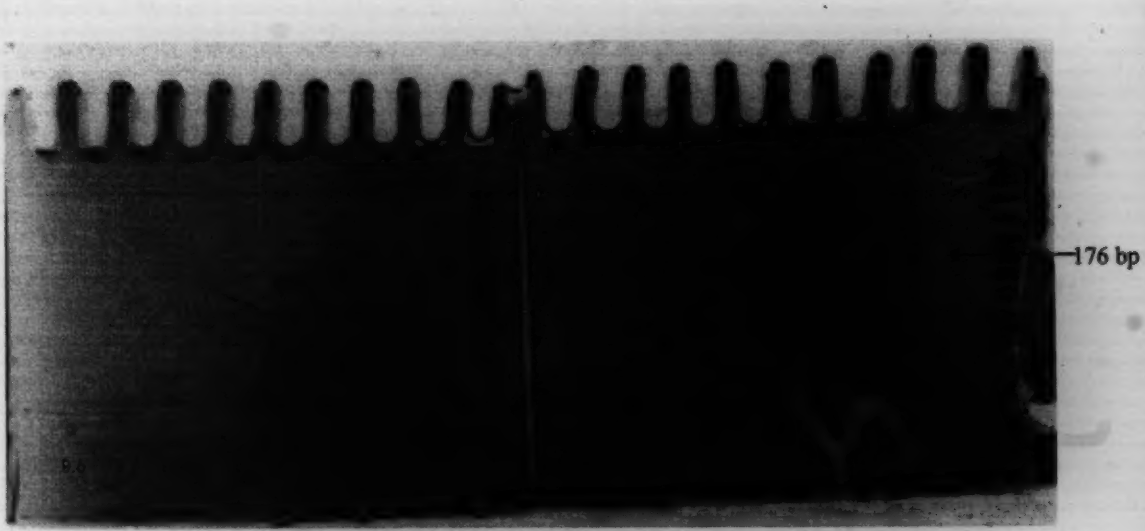


Figure 8.3 – PCR amplification of HVI products run on 10% polyacrylamide gel; Iberian Peninsula samples. Details of the reaction conditions can be found in section 7.7.

The samples were analyzed in two separate groups of ten teeth each; the first group of ten was partially analyzed with restriction enzymes, restriction fragment length polymorphism (RFLP) analysis, and shown to belong to at least two different haplogroups. There was not enough sample, extracted and amplified, to allow a complete determination of the sample's mtDNA haplogroup by restriction digestion analysis. But some information about the haplogroup of the samples could be gathered even without a complete restriction digestion analysis. Sample 9.16 is haplogroup H (Figure 8.4). Samples 9.7, 9.11 and 9.17 (Figure 8.4) are very likely haplogroup H but will require sequencing for confirmation. Sample 9.4 is neither haplogroup H nor haplogroup V. The high number of haplogroup H in this small sample is expected because haplogroup H is present in over 35% of the modern Portuguese population (Pereira *et al.* 2000). The restriction analysis was incomplete due to the limited amount of sample DNA available. There was no sequencing reactions carried out with these samples.

The second group of ten samples was analyzed using sequencing to determine the mtDNA haplogroup instead of restriction digestion. Sequencing requires less sample volume for analysis than restriction digestion analysis. The sequenced samples revealed DNA damage, sometimes to the extent that the haplogroups were difficult to assign.

J

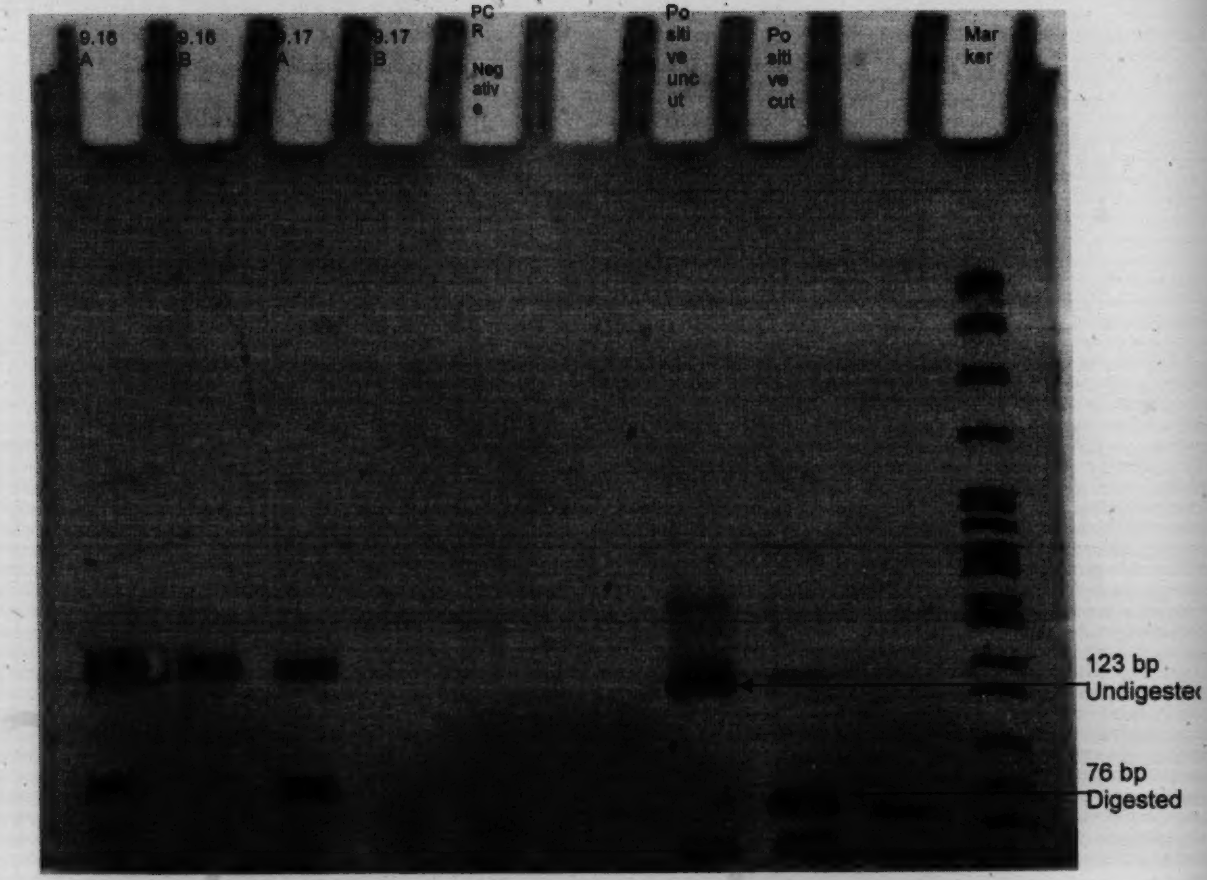

9.16 A
9.16 B
9.17 A
9.17 B
PCR Negative
Positive uncut
Positive cut
Marker
123 bp Undigeste
76 bp Digested

Figure 8.4 – Haplogroup H digestion of Iberian Peninsula Samples run on a 10% polyacrilimide gel. The 123 bp bands are uncut and indicate haplogroup H. The 76 bp bands had been digested and indicate the sample is not Haplogroup H.

Table 8.3 Iberian Peninsula Sample Summary

Sample Identification	Sample site	Age of Site (YBP)	Amplification?	Duplicate?	Haplogroup
9.2 (Arruda 42.1)	Arruda	Mesolithic 7224-7783	Yes	Yes	Unknown
9.3 (Arruda 42.2)	Arruda	Mesolithic 7224-7783	Yes	Yes	Not Haplogroup H
9.4 (Furninha F1)	Furninha	Neolithic 5500-6900	Yes	Yes	Neither H nor V. (Restriction Digestion)
9.5 (Furninha F2)	Furninha	Neolithic 5500-6900	Yes	No	Unknown
9.6 (Furninha F3)	Furninha	Neolithic 5500-6900	No	No	Unknown
9.7 (Furninha F4)	Furninha	Neolithic 5500-6900	Yes	No	H (Restriction Digestion)
9.8(Font 1)	Fontainhas	Neolithic 4724	Yes	No	Unknown
9.9 (Font 2)	Fontainhas	Neolithic 4724	Yes	Yes	H (Restriction Digestion)
9.11 (CdM2)	Casa da Moura	Neolithic 5595-6869	Yes	Yes	H (Restriction Digestion)
9.12 (CdM3)	Casa da Moura	Neolithic 5595-6869	Yes	No	Unknown
9.13 (CdM4)	Casa da Moura	Neolithic 5595-6869	Yes	Yes	Unknown
9.15 (CdM6)	Casa da Moura	Neolithic 5595-6869	Yes	Yes	V (Sequencing)
9.16 (Feteira1)	Feteira	Neolithic 4660-5297	Yes	Yes	H (Restriction Digestion)
9.17 (Feteira 2)	Feteira	Neolithic 4660-5297	Yes	Yes	H (Restriction Digestion)
GAC 240	La Garma	Mesolithic and Neolithic	No	Yes	U5a (Sequencing)
GAA 258	La Garma	Mesolithic and Neolithic	Yes (See Figure 8.3)	Yes	V/H (Sequencing)
GAC 421	La Garma	Mesolithic and Neolithic	Yes (See Figure 8.3)	Yes	V/H (Sequencing)
GAA 904	La Garma	Mesolithic and Neolithic	Yes	Yes	Unknown
ECM	El Cotero de la Mina	Neolithic	Yes	No	Unknown
Melides	Melides	Neolithic 4989-6144	Yes	No	Unknown

The dates for the sites are presented in Years Before Present (YBP) and were provided in Jackes *et al* 2001.

The PCR products from amplification of samples from 9.15 (CdM4), GAC 421, GAC 240 and GAC 258 were directly sequenced. Sample GAC 240 showed sequences that were typical of both haplogroup V and H. Sample 9.15 appears to be haplogroup V, the A sample shows a typical haplogroup V motif while the B sample shows too much damage to be typical of any haplogroup.

The sequences often did not agree between the A and the B sample, possibly due to the separate extraction and amplification of the A and B samples. Because there was no phylogenetic paradox present between the haplogroups of the researchers and the expected haplogroups from the samples, it is impossible to distinguish between the endogenous DNA and possible contamination by sequence alone; instead authenticity is derived from the adherence to the authenticity criteria that are enumerated in chapter 6.

The source of any potential contamination could not be determined; the haplogroups of the people that handled the teeth, M. J. (V), D. L. (H) and E.G. (H), do not match completely with the sequences seen in the samples and several of the researchers share mtDNA haplogroups. The samples are likely to contain a mixture of contamination and damage.

Table 8.4: Iberian Peninsula Samples
and assigned haplogroups

Sample		Polymorphisms	Haplogroup
GAC 258	A	16223G 16266T 16298C	V
	B	16295T	H
9.15	A	16298C	V
	B	16260G 16266G	H
GAC 421	A	16269T 16298C	V
	B	16266G	H
GAC 240	A	16256T 16263C 16269T	U5a
	B	16256T 16260G 16266G 16303T 16305T	U5a

The DNA amplified by PCR and sequenced is likely to be endogenous despite changes of some of the nucleotides. Some of the evidence for authenticity, aside from following the authenticity criteria, is that there were different haplogroups detected in the samples, and not all of the haplogroups detected match the haplogroups of the researchers. 74% of the PCR amplifications did not contain any signs of contamination as evidenced by the uncontaminated extraction blank and PCR negative controls. The amplifications that appeared contaminated, a product in the PCR negative or product in more than 60% of the lanes, were disregarded. These measures ensured that any products sequenced would have the greatest likelihood of representing endogenous DNA.

The sites that provided amplifiable DNA were Furninha, Casa da Moura, Feteira and La Garma. La Garma is a mixed Mesolithic and Neolithic site; the specific ages of the samples are unknown, while Furninha, Casa da Moura and Feteira are all Neolithic sites. There appears to be a possible correlation that the sites that yielded DNA were the younger Neolithic sites, rather then the older Mesolithic sites. However, this observation is skewed by the fact there was only one Mesolithic site included in the study, Arruda, and that La Garma is the only site with Mesolithic and Neolithic material.

The number of contaminated PCR amplifications was 5 out of 19 amplifications, a contamination rate of 26%. The two DNA extractions that the samples were divided into are left separate here to emphasize that there was no systemic contamination of samples during the DNA extraction to account for the contamination.

Extraction Batch	#1	#2	Total
Total Amplifications	8	13	21
Amplifications with a clean PCR negative	5	9	14
Contaminated Amplifications	1	4	5

Table 8.5 - PCR Amplifications for the Iberian Peninsula samples: contaminated and uncontaminated

8.4 Khuzhir-Nuge XIV

The demonstration of successful amplification of DNA from this cemetery was the main objective of this portion of the project. Because of the lack of success in extracting DNA from the bones of individuals in this cemetery, the best chance for the recovery of DNA was from the teeth. As many as three teeth were available from each individual; if the first tooth did not yield amplifiable DNA then the second tooth was mined. The third tooth was mined if there was no DNA detected in the first and second tooth.

There were 35 teeth sampled which came from 27 individuals, who were buried in 23 separate graves. All three available teeth were mined for 2 individuals, two of the available teeth were mined for 4 individuals, and the remaining 21 individuals had a single tooth mined. Three graves had two individuals sampled from them, graves 27, 58 and 59. A multiple grave is indicated when the grave number is followed by an individual number. For example 27.2 refers to grave 27 and the second individual in that grave. The second and third tooth samples were analyzed if there was no DNA detected in the first tooth sample.

There was duplicate amplification from eight of the samples (e.g. sample 1999.136A and 1999.136B show amplification in figure 8.5). Three of these samples were cloned and all eight of them were sequenced directly.

The three cloned samples had clones prepared from both the A and B products. The separate A and B clones were sequenced at the heavy and the light strand.

The sequences did not always agree between the heavy and the light strand. There was often disagreement between the A and the B product. This disagreement is explained by the fact that the A and the B samples were amplified separately from degraded templates. It is possible that there is any combination of authentic sequence and contamination when observing the A and B extracts for a single sample. In figure 8.5 there is an example of the duplication of amplification products in 1999.136 A and 1999.136 B. When examining the sequencing data for these products it is expected that there would be extraneous nucleotide polymorphisms detected. Damage to the DNA molecule will lead to the creation and subsequent detection of nucleotide polymorphisms that do not match a haplotype motif.

Table 8.6 Individuals, graves and teeth sampled from Khuzhir-Nuge XIV.

Grave and Individual No.	Sample No. of Tooth #1	Sample No. of Tooth #2	Sample No. of Tooth #3
7	1997.211	■	
9	■		
17	■		
27.2	■		
27.3	2003.633 (14)		
32	■		
33	2003.579 (17)		
35.1	1998.355	■	
36.1	1998.364	■	
37.1	1998.368	▨ 1998.369	
40	▨ 2003.588		
42	▨ 1999.174		
46	1999.131	■	▨ 1999.129
47	▨ 2003.599		
48	▨ 1999.159		
49	2003.604 (32)		
50	■		
51	1999.136		
55	1999.142		
58.1	■		
58.2	▨ 1999.169		
59.1	▨ 2003.593		
59.2	■	■	▨ 1999.172
65	▨ 2003.629		
68	▨ 2000.132		
74	▨ 2003.632		
80.2	2000.124		

□	Extracted Oct 2, 2004
■	Extracted Feb. 16 and April 7, 2005
▨	Extracted Oct. 26 and Nov. 22, 2005

27 Individuals Sampled
3 Graves with multiple individuals had
2 people sampled
35 Teeth

2 Individuals had 3 teeth mined
4 Individuals had 2 teeth mined
21 Individuals had 1 tooth mined

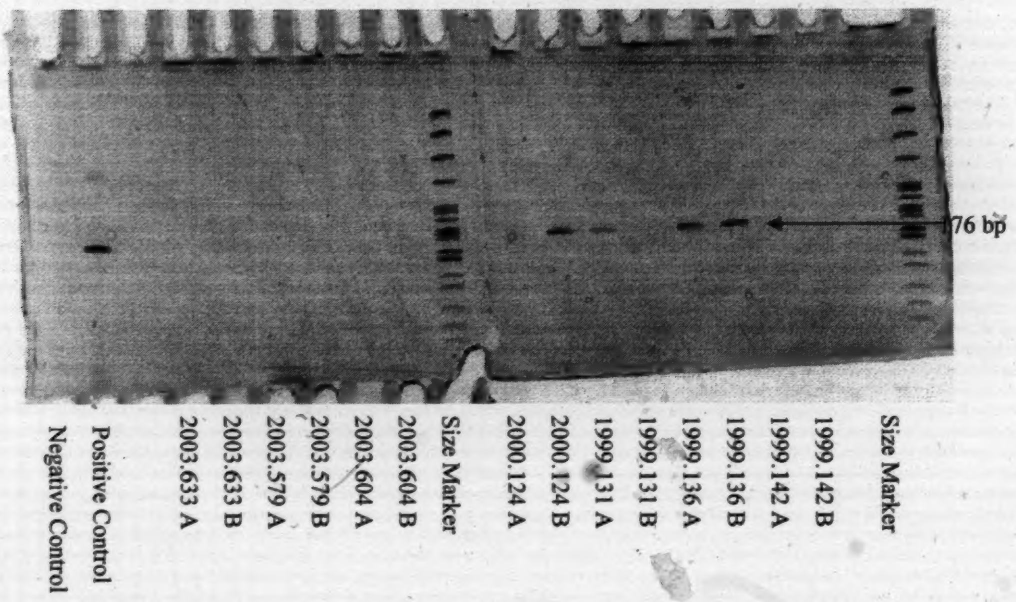

176 bp
Negative Control
Positive Control
2003.633 A
2003.633 B
2003.579 A
2003.579 B
2003.604 A
2003.604 B
Size Marker
2000.124 A
2000.124 B
1999.131 A
1999.131 B
1999.136 A
1999.136 B
1999.142 A
1999.142 B
Size Marker

Figure 8.5 – Khuzhir-Nuge XIV Samples PCR amplification of HVI products run on a 10% polyacrylamide gel. The primers and conditions used are listed in detail in section 7.7 PCR.

Table 8.7: Khuzhir-Nuge XIV Samples and Haplogroups. The polymorphisms were gathered from sequencing information. The haplogroups were determined

Sample		Polymorphisms	Haplogroup
2003.604	A	16298C	V
	B	16298C	V
	Clone A2	16298C	V
	Clone B2	16271C 16278T 16293G 16311C	damage
1999.136	A	16298C	V
	Clone A3	N/A	H
	Clone A7	N/A	H
	Clone B7	N/A	H
1999.142	A	N/A	H
	B	16298C	V
	Clone A7	N/A	H
	Clone A8	N/A	H
	Clone B7	16220G 16298C	V
1999.153	A	16263C 16265T 16269G 16298C	V
	B	16260G 16266G 16269T 16290A 16291G 16297G 16299G 16324A	damage
1999.173	A	16298C 16311C	V
	B	16231C 16256T 16267T 16270T	Possible U5a
1997.220	A	16270T	Possible T
	B	16294T 16296T	Possible T
1998.347	A	16227G 16304C	H
	B	N/A	H
1998.363	A	16270A 16291A	Possible U5a
	B	16260G 16283G 16294T 16295T 16296T 16326G 16329G	damage

There was contamination of the Siberian samples with both H and V Caucasian haplotypes. Often H and V were seen in a single sample when the A and B extractions were compared. The presence of differing contamination patterns for the same sample suggests that the results were contamination. This might have occurred at the stage of PCR amplification rather than during the DNA extraction; if contamination occurred during DNA extraction it would be expected that the sample would have the same contamination rather than a mixture. If all the samples in an extraction batch were contaminated at the same time during the extraction step, then the pattern of contamination would show the same haplogroup in all of the samples. There were at least three people that handled the samples between excavation and analysis. The people that worked in the ancient DNA laboratory had the same mtDNA haplogroups as the identified contamination. Due to the presence of multiple researchers with the same mtDNA haplogroup without dedifferentiating polymorphisms in the sequence studied, it was not possible to identify the person who was the source of contamination. 78% of PCRs performed had no contamination and no products (Table 8.8); there was a contamination rate of 22%. Because of the large proportion of PCRs with no contamination and no PCR products from the samples it is likely that there is very little DNA present in these teeth. The conditions at Khuzhir-Nuge XIV did not allow for DNA in teeth or bones to be preserved.

Extraction Batch	Oct. 2, 2004	Feb. 16, 2005 April 7, 2005	Oct. 26, 2005 Nov. 22, 2005	Total
Total Amplifications	11	11	10	32
Amplifications with a clean PCR negative	8	9	8	25
Contaminated Amplifications	3	2	2	7

Table 8.8 - PCR Amplifications for Khuzhir-Nuge XIV: clean and contaminated

Chapter 9: Conclusions

9.1 Overview

The analysis of ancient DNA requires clean consistent extraction protocols and high fidelity PCR amplification and sequencing before any results can be regarded as authentic. There are challenges with aDNA analysis that need to be overcome to produce clean, consistent results. The challenges are: contamination, fragmentation of the DNA molecule, mutation introduced by damage and how to minimize the impact of each of these challenges. The DNA in teeth has potentially better preservation than the DNA in bone. However, previous methods of extracting DNA from teeth were destructive; to work with teeth in a non-destructive manner a new tooth mining method was developed and then tested on modern teeth. Having this method confirmed, it was then possible to use teeth as a source of DNA to gain information about past cultures. In particular the Serovo-Glaskovo during the Neolithic period and that of the Iberian Peninsula during the Mesolithic-Neolithic transition.

9.2 Mining Method

Teeth are valuable for the biological and anthropological information that they contain. The shape of the tooth can provide information about which population they belonged to and tooth wear can tell you about the diet a person ate, the enamel of the tooth can be used for isotope analysis and show where the person grew up while the cementum rings can tell about how old the person was when they died. The DNA data can be reached without destroying the other information that a tooth can provide by using a non-destructive mining method.

The mining method described earlier in this thesis accomplishes the purpose of allowing DNA to be extracted from the interior of the tooth with minimal contamination, while at the same time not causing damage to the structure of the tooth. There are refinements that can still be made to the mining technique that will improve the usefulness of this protocol even more.

During the mining of the tooth when a rotary tool is used there is a degree of aerosolization that occurs, increasing the risk of cross-contamination between samples; a lower drill speed is not an option when cutting into the tooth due to the hardness of a tooth. This study used an enclosed Plexiglas box to contain the sample during processing; there was no airflow into or out of this box to prevent contamination of the ancient sample with modern DNA or cross-contamination of other ancient samples from aerolsolization. A solution to this issue is the use of a dedicated laminar flow hood; however, it was shown in another study in our laboratory that the use of a shared laminar flow hood was responsible for contamination. Therefore a dedicated flow hood would be the optimal solution to minimize contamination from aerolsolization.

One of the areas of improvement that was noted is that in the cases where the teeth were particularly fragile there was a substantial risk of shattering the tooth from the manipulation needed to accomplish mining of the dentine. Better methods of holding the tooth securely and safely need to be explored until an alternative that can hold and cushion fragile teeth can be found. In addition the choice of teeth that can be used in mining should be restricted to firm, well preserved teeth.

9.3 Modern Teeth

The new method of mining DNA from teeth without destruction was successfully developed with modern teeth. There was sufficient DNA in the dentine powder for extraction and amplification; subjective analysis showed that mining the interior of the tooth was as effective as grinding the whole tooth. These samples were modern and expected to have robust DNA present in them that would amplify strongly; the presence of robust modern DNA increases the possibility of cross-contamination between the samples, but during amelogenin typing no cross-contamination was observed. There was a success rate of 97%, 30/31 teeth had amplifiable mtDNA, when amplifying mtDNA. Amplification of the amelogenin gene on the sex chromosomes had a success rate of 58% (18/31 teeth had amplifiable nuclear DNA) There was no contamination detected and none was expected while working with modern teeth. Working with modern teeth that had robust non-degraded DNA was ideal conditions for the extraction of DNA from teeth, but the circumstances show that extractable, amplifiable DNA can be recovered from teeth without destruction or contamination.

The amplification products from the modern teeth were subjectively evaluated by two researchers at separate times for amplification strength. T-tests were used to determine if there was a difference in amplification strength between the teeth that had been exposed to factors that could have been detrimental to the recovery of viable DNA.

For mtDNA both researchers agreed that there was no difference in amplification strength between teeth that had been mined and teeth that had been ground. It was also agreed that there was no difference between teeth that had fillings and those that had no fillings. There was no agreement about the significance of the effect of a root canal and X-ray exposure on the amplification strength of mtDNA.

When assessing nuclear DNA amplification the two researchers agreed that there was a difference in amplification strength when the tooth had a filling or had been exposed to X-ray radiation. There was no difference between the amplification strength of teeth that had a root canal done and teeth that were had not had a root canal. A comparison of the amplification strength of teeth that had been mined to teeth that had been ground showed both that there was a difference in amplification and no difference.

The second researcher was not able to assess the amplification strength of teeth that had been submerged in bleach for 62 days. On the basis of a single researcher assessing the amplification strength there was an adverse effect on amplification for both mtDNA and nuclear DNA when the teeth have been submerged in 30% hypochlorite for 62 days.

9.4 Iberian Peninsula

Having successfully tested the mining technique using modern teeth, the new protocol was then tested with ancient teeth. Teeth from Mesolithic and Neolithic sites in Spain and Portugal were chosen for testing. There was a success rate of 90% for amplification of DNA from the twenty tooth samples. However, the success rate of amplifying in duplicate was 60%.

Where there was successful amplification in duplicate of the samples, they were subsequently sequenced. The haplogroups determined from sequencing were haplogroups H and V, typical European haplogroups. The researchers at the laboratory were found to be either haplogoup H or haplogroup V. Expecting to find the same haplogroups in the samples and among the researchers means that there was no phylogenetic paradox present. The researchers did not have any distinguishing polymorphisms that matched the polymorphisms seen from the samples. Because of this it is difficult to tell if there was contamination or not. The sequences of the samples look like the sequences of the researchers, but this is what was expected. It is impossible to say with confidence that the sequences from the samples are either endogenous or contamination. By having satisfied many of the criteria for authenticity it is possible to say that it is very likely that the sequences amplified from the samples are authentic, but it is not certain.

The sequence information gathered concurs with the haplogroup data known about the Mesolithic and Neolithic people of Spain and Portugal. The Mesolithic samples have shown the haplogroups U, U5, and H, while the Neolithic shows haplogroups U, U5, H and V (Chandler *et al* 2005). The sample size of this study, four sequences, is too small to allow any conclusions to be drawn about the frequency of haplogroups in either the Mesolithic or Neolithic population. The samples that yielded haplogroup information were from the Neolithic or were mixed Neolithic and Mesolithic, and the haplogroups seen were U5a, H and V. This is consistent with the results of other studies. Both within this study and that of Chandler and colleagues there is no evidence of haplogroup J. As a relatively recent addition to the populations of Portugal, the lack of haplogroup J in both the Mesolithic and Neolithic populations indicates that the introduction of farming in the region was not concurrent with the introduction of haplogroup J from the Near East. It is likely that farming was introduced to the Iberian Peninsula by a different method.

The Neolithic site, Furninha, has been analyzed for collagen content and it was shown that there was no collagen in the sampled bone (personal communication with Dr. Jackes and Dr Lubell). Sample 9.4, from Furninha, amplified in duplicate and without evidence of contamination. The RFLP analysis indicated that the haplogroup was neither H nor V. Due to the small volume of sample from teeth there was no further analysis and the haplogroup was not determined. Because many of factors that degrade collagen also degrade DNA, it would be unlikely that there was DNA when no collagen is present in samples from the same site. If the haplogroup of sample 9.4 had been the same as the researchers, then it could have been dismissed as contamination. But the presence of a haplogroup that is not contamination from the researchers indicates that there was DNA preservation in the teeth despite the lack of collagen in bone samples from the same site.

The tooth samples from Spain and Portugal have demonstrated that the mining technique is suitable for ancient samples as well as modern samples. Analysis of more samples will be required to reach any conclusions about the introduction of agriculture to the Iberian Peninsula.

9.5 Khuzhir-Nuge XIV

The retrieval of DNA from the ancient Iberian Peninsula teeth samples demonstrated that the mining technique could be applied to other ancient samples. Mining was then used to remove dentine from the ancient samples from Khuzhir-Nuge XIV and DNA was extracted and amplified. Of the 34 teeth mined, representing 27 individuals, there was a 24% success rate for samples that amplified in duplicate. When the samples that are obvious contamination are treated as an unsuccessful amplification then the success rate drops to a 9%. The eight successful amplifications represent separate individuals. The criteria for authenticity were followed and due to the presence of a phylogenetic paradox between the Asian skeletal remains and the predominately Caucasian researchers, it was also possible to determine if the products sequenced were due to contamination rather than endogenous DNA by their determined haplogroup. The samples from Khuzhir-Nuge showed evidence of contamination after sequencing due to the detection of Caucasian mtDNA haplogroups where only Asian mtDNA haplogroups were expected. Where there was no contamination the degradation of the sample was so great that the determination of a haplogroup was impractical. This difference in the expected haplogroups and the haplogroups of the researchers set up a phylogenetic paradox that allowed the identification of contamination. The contamination detected of the samples was with both H and V haplotypes; there are examples of the typically European haplogroup H occurring in Southeastern Siberia (Derenko *et al* 2003), but these examples of haplogroup H are very rare; therefore, for the purposes of this study all haplogroup H sequences will be regarded as contamination.

It is important to determine at what stage in handling the teeth contamination occurred in order to prevent contamination during further research; there are several times when contamination of the samples with modern DNA may have happened: handling prior to mining, during mining, during DNA extraction and during PCR amplification.

There were an unknown number of people that handled the samples during the excavation and curation of the teeth, at least one person handled the teeth during excavation and another worked with the teeth for isotopic analysis, this researcher was determined to be mtDNA haplogroup K and was not identified as a source of contamination. At least four people were working in the same laboratories during the period of this study; two of those four researchers are known to be haplogroup H and the other two researchers are haplogroup V (Table 9.1). and distinguishing polymorphisms, where present, were insufficient to allow the identification of specific individuals as the sources of contamination.

Table 9.1: Polymorphisms and assigned haplogroups of laboratory personnel

Personnel	Polymorphisms	Haplogroup	
E.G.	16273 C 16280 T 16281 G	H	Worked with all samples
F.B.	N/A	H	Present at laboratory, did not handle samples
K.M.	16298 C	V	Used laboratory equipment, did not handle samples
T.T.	N/A	H	Used laboratory equipment, did not handle samples.
C.D.	16298 C	V	Used laboratory equipment, did not handle samples
A.L.	N/A	H	Collected teeth at KNXIV. Used gloves.
C.H.	16224 C 16311 C	K	Handled KNXIV teeth for examination. Used gloves and a mask.
D.L.	N/A	H	Handled Iberian samples
M.J.	16298 C	V	Handled Iberian samples

There is the possibility that contamination could have been introduced at any point in the process of working with samples. The samples were collected by students during an archaeological field school. Though the teeth samples were not initially intended for DNA analysis, precautions, such as wearing disposable latex gloves, were used. The sample teeth were quickly placed in sample bags, sealed, and stored for up to six years at room temperature. When Kemp and Smith (2005) left a purposefully contaminated sample exposed to air for a much shorter period of time, the contaminating DNA had been degraded and was undetectable. However, when purposefully handled samples were analyzed for contamination by Mooder (2004) she found that the most prevalent contamination was that of an osteologist who had handled the materials eight years previously rather than the individual who had handled the materials immediately before analysis. The length of time that detectable DNA can survive on a sample is unknown and is likely to be dependent upon the individual handling the samples as well as the materials themselves.

After the teeth were removed from storage extensive precautions were taken during the handling and analysis of the teeth to prevent contamination with modern DNA. Contamination may potentially occur any time, such as during handling and sample manipulation, there is potential for contamination to occur and precautions must be taken. This included the use of dedicated laboratories and the wearing of double gloves when working with samples. There were multiple controls associated with the samples during extraction and PCR amplification. During extraction a negative extraction blank was treated as another sample and underwent the same DNA extraction procedure with the other samples and during the PCR amplification there was both a negative PCR control and a positive PCR control. Each ancient sample was amplified multiple times, if the negative controls, extraction and PCR, showed contamination, then those amplifications were discarded.

A portion of the tooth crown was excised for use in isotopic analysis by another researcher, C.H. As a part of the precautions taken by researchers in the laboratory and researchers who were known to have handled samples had a sample of DNA extracted and typed for their haplogroup. The researcher C.H. was determined to be haplogroup K. Gloves were worn while the teeth were being handled by C.H. as this handling is potentially a source of contamination. However, the haplogroup K was not found as contamination when the sample sequences were examined; therefore, the precaution of wearing gloves when working with tooth samples prior to DNA analysis may be sufficient to prevent contamination with modern DNA. The handling of the samples for removal of a piece of the crown for isotopic analysis is not a source of contamination in this study.

The mining procedure itself is a potential source of contamination. The precautions taken to prevent contamination were to wear: a mask, double set of gloves, hair net, disposable sleeves and full body suit with hood. The author is haplogroup H, but contains several non-informative polymorphisms; the contaminating sequences do not contain the full motif that would match the haplogroup of the author. As the author is the only researcher to have handled the teeth during mining and the author is not the source of contamination, then it is very likely that contamination did not occur during mining.

Some of the mining instruments were used for several samples, allowing for the possibility of cross contamination. Precautions taken to prevent this included a combination of bleach cleaning of the tools to oxidize potentially contaminating DNA, autoclaving, which used a combination of heat, moisture and pressure to degrade DNA. All three of these precautions were taken to ensure the shared equipment was contamination free. An examination of the haplogroups present as contamination show that cross contamination between samples was not an issue in this study. The only contaminating sequences detected were of European origin and not of Asian origin; if there was cross contamination between samples there would be Asian haplogroups detected. However there was not reliably authentic Asian DNA detected either, so it is difficult to determine if the precautions were sufficient to prevent cross contamination between samples. Therefore the sharing of some instruments between samples during the mining procedure is not likely to be the source of contamination.

There was an extraction blank used as a negative control during the process of DNA extraction. In all cases during this extraction blank showed no contamination when amplified. Therefore it is unlikely that there was any contamination introduced at the point in the experiment where DNA was extracted from the mined tooth powder. If there was contamination present in the sample before the extraction step, then it would be possible to have a contaminated sample and a clean extraction blank. Additionally there were different types of contamination detected within the same extraction, suggesting that either there were multiple sources of contamination or that the contamination occurred at a later time.

The extremely sensitive PCR amplification remains as the most likely time during which contamination could be introduced; the setup for the PCRs was carried out in the dedicated ancient DNA laboratory where there were other researchers working with their samples throughout the period of the study. Even if a laboratory was to be working with a single sample set at a time there is still a possibility of contamination from other samples or from the researcher. The number of template molecules of DNA that are expected to be extracted from an ancient sample is very low; it is very likely that at any point during the PCR setup that a small amount of contamination could have occurred and the PCR amplification would preferentially amplify the undamaged modern contaminating DNA. The result is an authentic looking product that would not be detectable as contamination until the products were sequenced. To reduce the possibility of introducing contamination to the extracted sample DNA, the DNA extract was handled as little as possible. It is possible that during the course of the 32 PCR amplification reactions encompassing 34 teeth that there was spurious contamination in some of the extracts.

The sequencing reaction usually shows the nucleotides of the most prevalent sequence in the mixture; there may be authentic DNA in the sample, but the presence of contamination will make this DNA unobservable. Conversely, it is similarly impossible to guarantee that there is no contamination in a sample that is being sequenced; all that could be said is that the most prevalent portion of the mixture appears to be authentic.

There was contamination present during the cloning of the samples; it is likely that the contamination was introduced at the PCR amplification step. During cloning each *E. coli* internalizes separate pieces of DNA and when that clone is sequenced only that piece of DNA will be seen. This way if the clones are sampled there will a clone representing each species of PCR amplification product that is in the PCR amplification mixture. When the clones of sample 199.136 were sequenced, it was discovered that there were two distinct haplogroups among the clones, haplogroups H and haplogroup V. This suggests that there was a predominant contamination with haplogroup V, which was observed during direct sequencing of the PCR products, while haplogroup H, which was not seen in the direct sequencing, and comprised a smaller portion of the contaminating DNA that was detected when a clone was sequenced.

The source of contamination could not be determined due to a lack of identifying polymorphisms; however, the stage during which contamination was likely to have occurred is during the highly sensitive PCR amplification stage. The precautions to prevent contamination will continue to be used, with new additions as new precautions are developed, and checks such as a phylogeographic paradox will be designed into future studies.

Despite the success of the mining method on other samples the teeth of Khuzhir-Nuge XIV yielded no amplifiable endogenous DNA. Due to the lack of success in retrieving DNA from bone samples collected from this cemetery over the last six years it is not surprising that there was no amplifiable DNA detected in the teeth.

9.6 Overall Conclusion

The method of mining teeth worked well with modern and ancient teeth. There is less material available for analysis when working with teeth instead of bone, but the possibility of recovering amplifiable DNA is greater. This method was proven to work with modern teeth that had undergone various treatments, as well as with ancient teeth.

It is unlikely that there is much amplifiable DNA to retrieve from the cemetery of KNXIV, either in the bones, from which there has been no DNA amplified despite numerous attempts, or in the teeth. It is possible that there is DNA present in the samples, but in a state in which the DNA is not accessible for amplification, such as when DNA is incorporated into melanoidine, by the Maillard reaction. Using protocols that incorporate PTB into the extraction procedure may release the DNA from the melanoidine and allow researchers to successfully amplify the DNA present in the sample.

The amplification of DNA from the Iberian Peninsula demonstrated that it was possible to amplify DNA from samples that are from around 7700 YBP. More work in the area is possible and informative. While the amplification of some DNA from Khuzhir-Nuge XIV indicated that it is possible to extract DNA from teeth at a cemetery where there was no amplifiable DNA from the bone, but the better the preservation of DNA in bone the more likely it is that the DNA preserved in the teeth is informative.

The mining technique works well and given samples of a sufficient degree of preservation it would be possible to use this mining method to retrieve aDNA from sample without destruction of the tooth.

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Chapter 8: Results

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Chapter 9: Discussion

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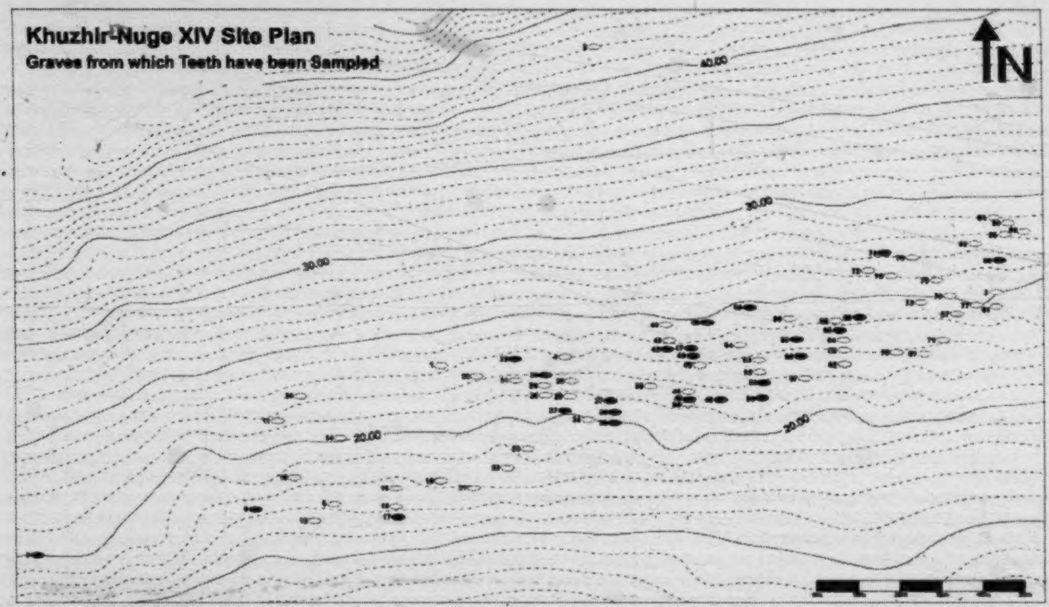
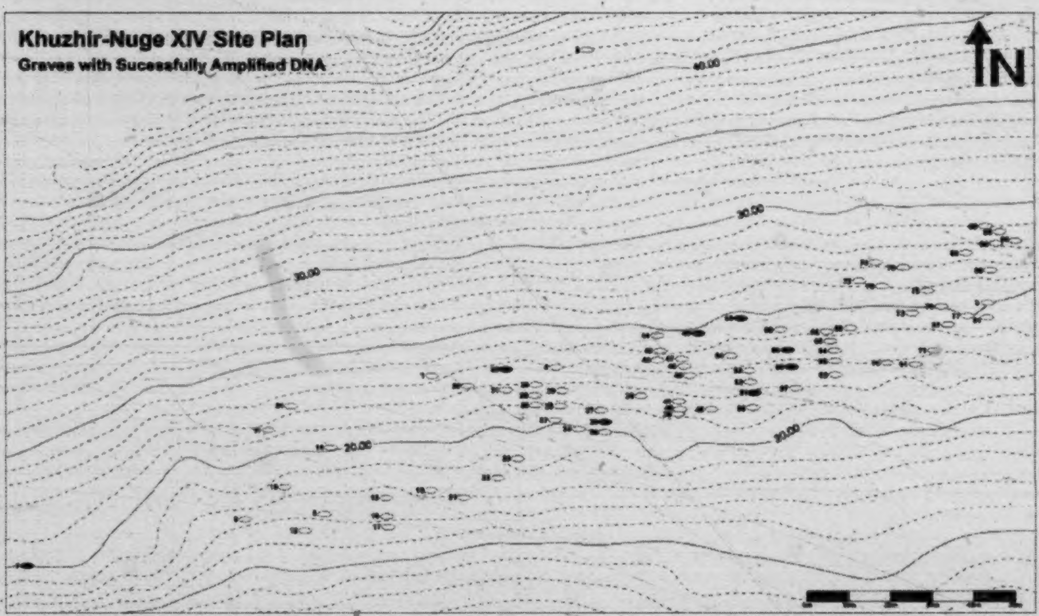
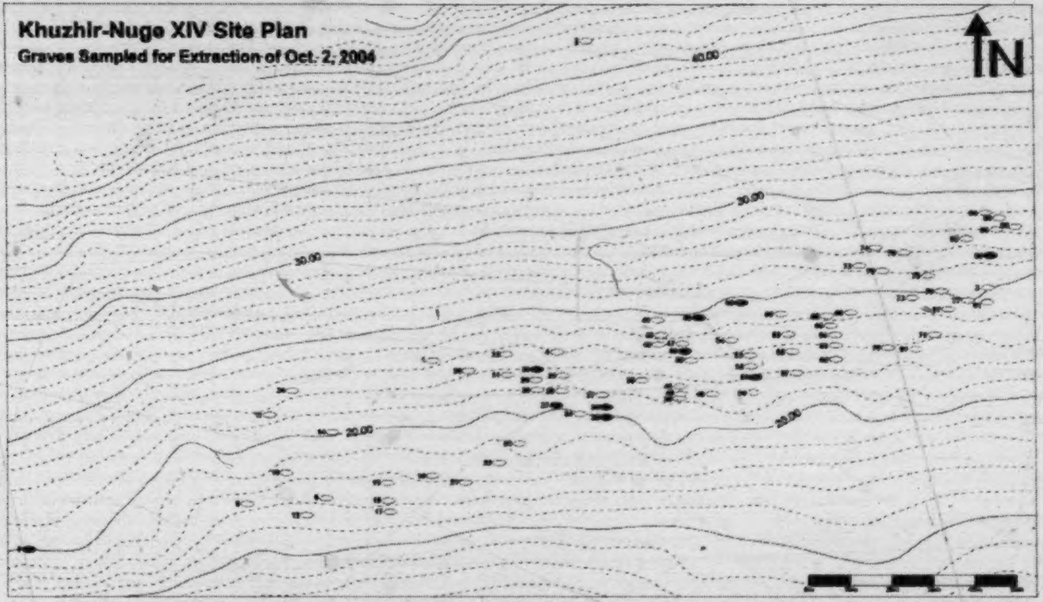
Appendix 1: Maps of Khuzhir-Nuge XIV grave sites


Khuzhir-Nuge XIV Site Plan
Graves from which Teeth have been Sampled
N
40.00
30.00
30.00
20.00
20.00

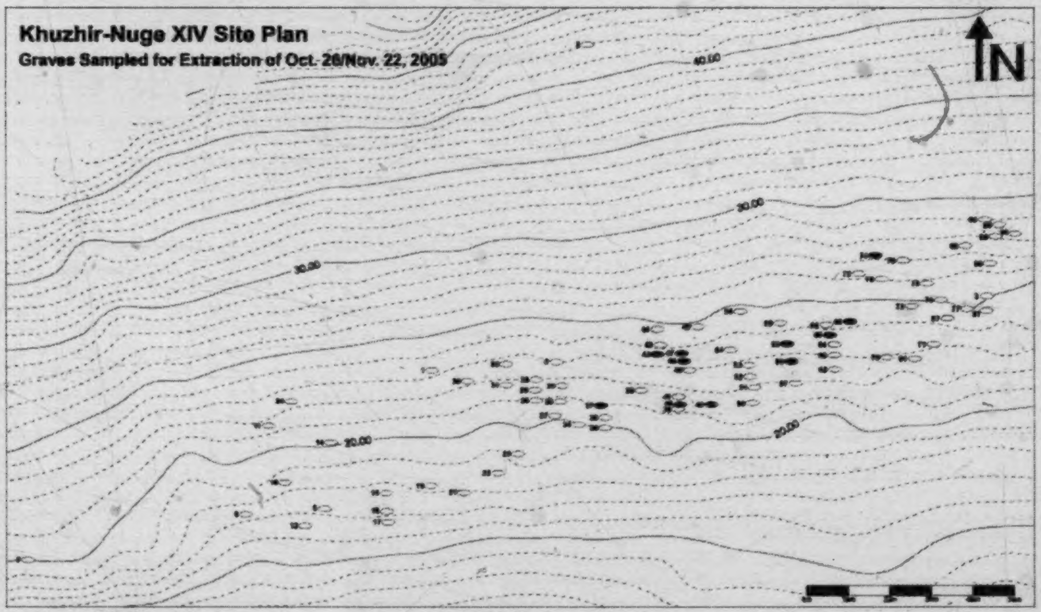
This site plan shows the location of the graves from which teeth were sampled at Khuzhir-Nuge XIV.


Khuzhir-Nuge XIV Site Plan
Graves with Sucessfully Amplified DNA
N
40.00
30.00
30.00
20.00
20.00

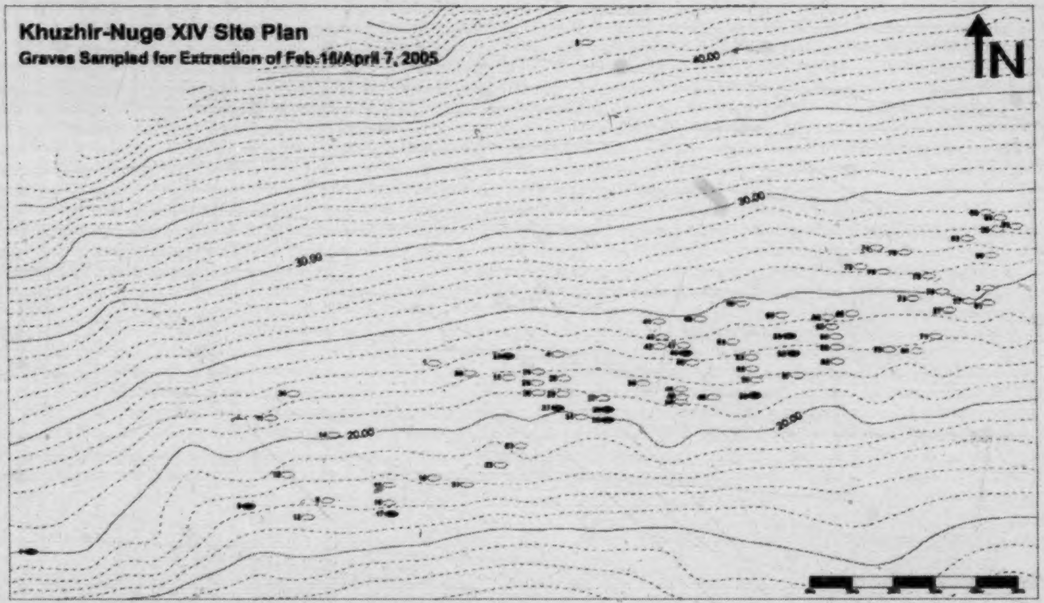
The graves from which the sampled teeth provided amplifiable DNA are indicated in black.


Khuzhir-Nuge XIV Site Plan
Graves Sampled for Extraction of Oct. 2, 2004
N
40.00
30.00
30.00
20.00
20.00

The teeth from the graves indicated in black were processed on October 2, 2004.


Khuzhir-Nuge XIV Site Plan
Graves Sampled for Extraction of Oct. 26/Nov. 22, 2005
N
40.00
30.00
30.00
20.00
20.00

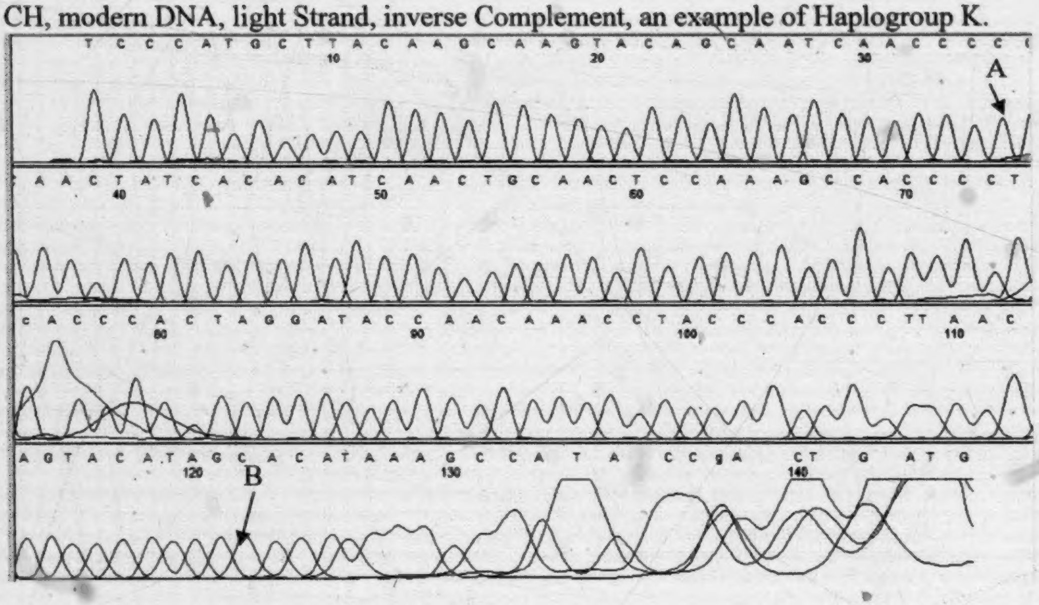
The teeth from the graves indicated in black were processed on October 26 and November 22 of 2005.


Khuzhir-Nuge XIV Site Plan
Graves Sampled for Extraction of Feb.16/April 7, 2005
N
40.00
30.00
30.00
20.00
20.00

The graves indicated in black show the graves from which teeth were processed on February 16 and April 7 of 2005.

Appendix 2: Representative Samples of HVI Sequence Analysis

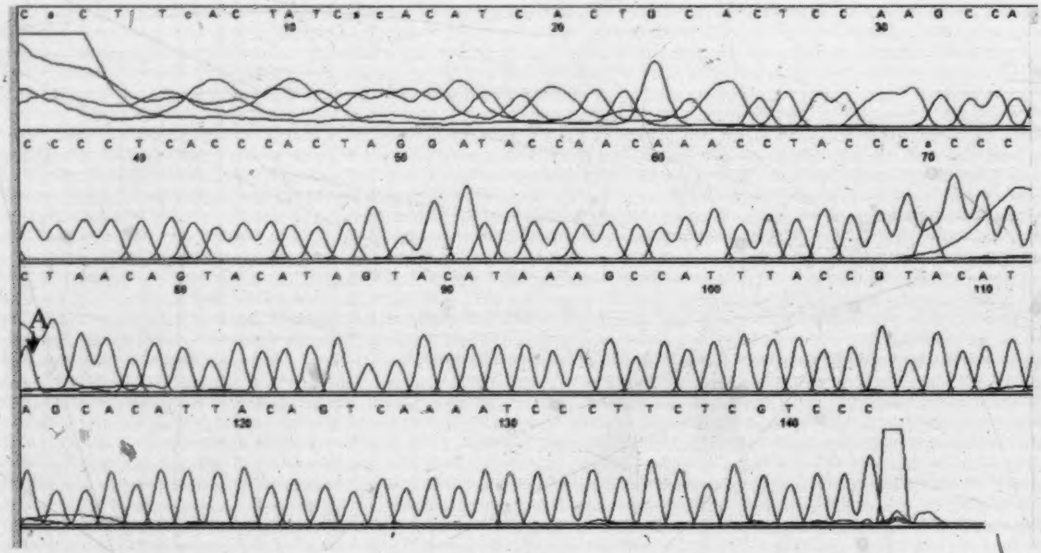
CH, modern DNA, light Strand, inverse Complement, an example of Haplogroup K.



A – 16311 C
B – 16224 C

The Light strand of DNA is the reverse complement of the Cambridge Reference Sequence; the haplotypes are given in terms of the heavy strand. CH is a researcher that handled some of the Siberian samples and is representative of Haplogroup K. All letters in the lower case indicate manual base calling.

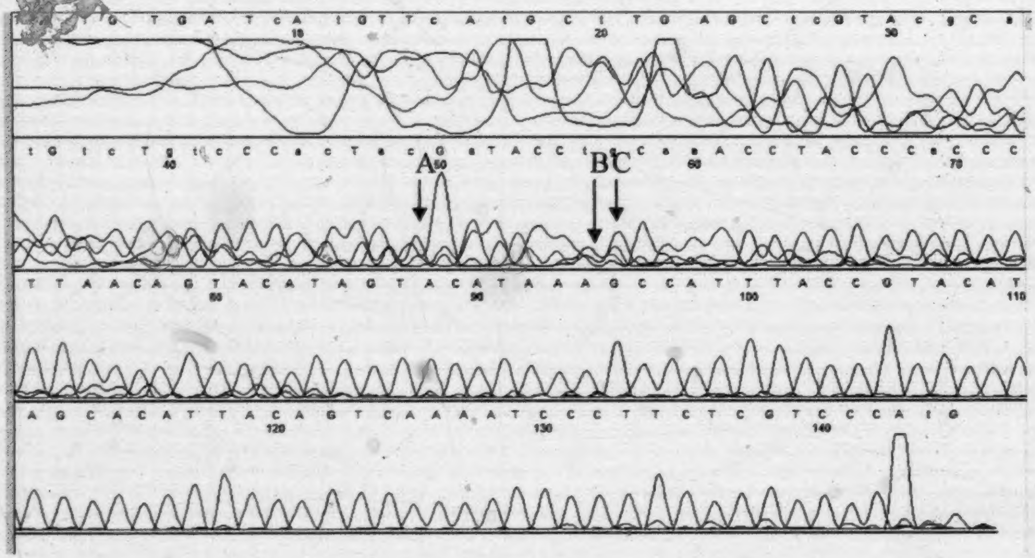
MJ, Modern DNA, Heavy Strand, an example of Haplogroup V.



A – 16298 C

MJ is representative of Haplogroup V and has worked with the Iberian Peninsula samples. All letters in lower case indicate manual base calling.

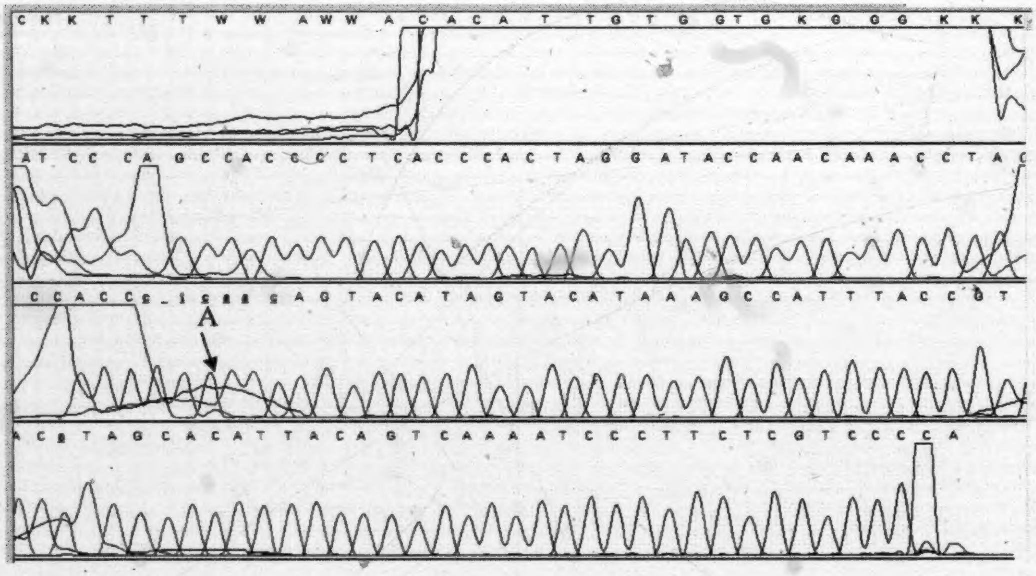
EG, Modern DNA, Heavy Strand, an example of Haplogroup H.



EG is typical of haplogroup H, there is concordance with the Cambridge Reference Sequence; the polymorphisms present are not indicative of another haplogroup. This researcher worked with the Iberian Peninsula samples and the Siberian samples. All letters in lower case indicate manual base calling.

A – 16273 C
B – 16280 T
C - 16281 G

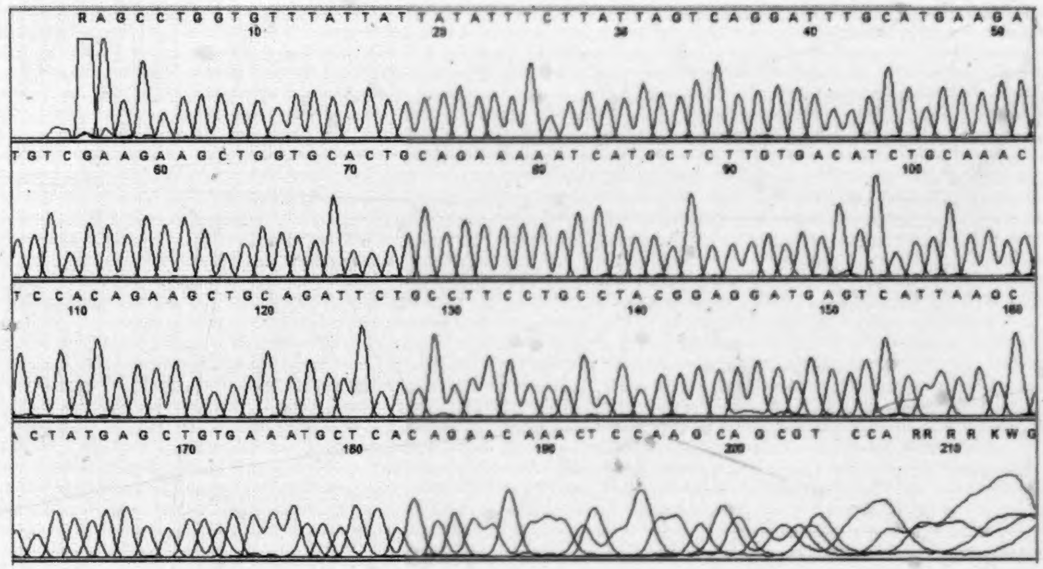
Sample 9.15 B, Iberian Peninsula, Neolithic Casa de Mora, Heavy Strand, and Haplogroup V.



A – 16298 C

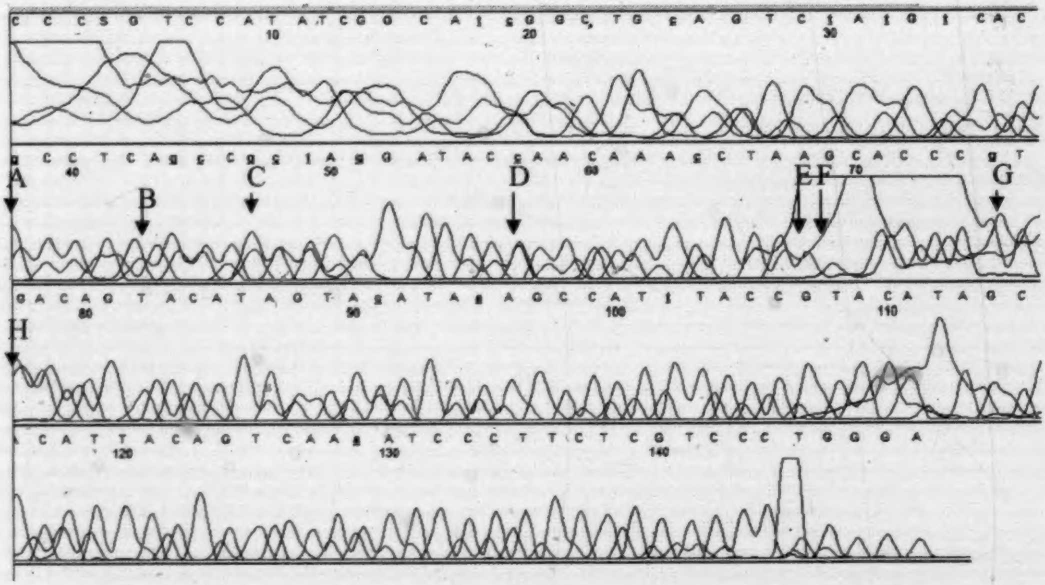
Sample 9.15 B is the second extraction of sample 9.15, this tooth came from the Neolithic site of Casa de Mora in the Iberian Peninsula. The degradation associated with ancient samples makes identification of haplogroups challenging. All letters in lowercase indicate manual base calling.

Sample 1998.347 B, Khuzhir-Nuge XIV, light strand, inverse complement, and Haplogroup H.



Sample 1998.347 B is the second extraction of sample 1998.347, this tooth came from the Khuzhir-Nuge XIV. The sequence seen here has no variation from the Cambridge Reference Sequence and is haplogroup H, finding haplogroup H at Khuzhir-Nuge XIV is indicative of contamination. All letters in lowercase indicate manual base calling.

Sample 1999.153 B, Khuzhir-Nuge XIV, heavy strand and unassignable haplogroup.



Sample 1999.153 B is the second extraction of sample 1999.153, this tooth came from the Khuzhir-Nuge XIV. The sequence seen here has several variations from the Cambridge Reference Sequence, but none of these polymorphisms are informative, instead they are likely to be due to damage to the DNA. All letters in lowercase indicate manual base calling. Base D shows two peaks of equal size for cytosine and guanine, this is indic[illegible]e of the presence of more than one species of PCR product being present in the mixture; the presence of more than one species is most likely due to contamination rather than heteroplasmic individual. The polymorphisms listed here are non-informative, indicative of contamination and damage rather than a haplogroup. The CRS (Cambridge Reference Sequence) nucleotides are shown in brackets after the polymorphisms are listed.

A – 16260 G (C)
B – 16266 G (C)
C – 16269 G (A)
D – 16279 C/G (C)
E – 16290 A (C)
F – 16291 G (C)
G – 16297 G (T)
H – 16299 G (A)